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Departamento de Bioquímica y Biología Molecular I

REGULACIÓN DE LA RESPUESTA INMUNE INNATA POR LA PROTEÍNA DE MATRIZ EXTRACELULAR TROMBOESPONDINA-1

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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REGULATION OF INNATE IMMUNE RESPONSES BY THE EXTRACELLULAR MATRIX PROTEIN THROMBOSPONDIN-1

REGULATION OF INNATE IMMUNE RESPONSES BY THE EXTRACELLULAR MATRIX PROTEIN THROMBOSPONDIN-1

A THESIS DISSERTATION SUBMITTED TO THE DEPARTMENT

OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

UNIVERSIDAD COMPLUTENSE DE MADRID

BY

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Bethesda

Dedicada a las niñas de mis ojos, María y Beatriz. A mi Juanma, a mi hermano Ricardo y a mis queridos padres.

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(**Gema Martin-Manso**, Susana Galli, Lisa A. Ridnour, Maria Tsokos, David A. Wink, and David D. Roberts. Thrombospondin1 promotes Tumor Macrophage Recruitment and Enhances Tumor Cell Cytotoxicity of Differentiated U937 Cells. *Cancer Res*. 2008; 68(17): 7090-9)

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FE DE ERRATAS

En el capítulo I de la Introducción (versión en Español), apartado A.1.1.3.de Conexiones Moleculares (*página 12*) aparecen unas referencias cambiadas. Las referencias correctas son las siguientes:

α3β1, α6β1, α9β1 y α4β1 (3, 58-60)
Los TSRs contribuyen a la unión de heparina con múltiples sitios de baja afinidad (55, 56)
CD36 (62,63)
Integrinas β1 (64, 65); *también en la pagina 17*, **integrinas de tipo β1** (64)
Decorina (57)
LRP (61)

Abbreviations

ADAMTS	proteins containing a disintegrin and a metalloproteinase with TSPs motifs
BSA	albumin bovine serum
Ca^{2+}	calcium
COMP	cartilage oligomeric matrix protein
CTL	cytotoxic T lymphocyte
DAB	3,3–diaminobenzidine
DCs	dendritic cells
ECM	extracellular matrix
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblast growth factor
FMLP	n-formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte-macrophage-colony-stimulating factor
HBD	heparin-binding domain

HBSS	Hanks' balanced salt solution
HSPG	heparan sulphate proteoglycans
H_2O_2	hydrogen peroxide
ICAM-1	intracellular cell adhesion molecule-1
IFNγ	interferon-γ
IL	interleukin
LAP	latency-associated protein
LDL	low density lipoprotein
LFA	leukocyte function associated antigen
LPS	lipopolysaccharide
LRP	LDL receptor-related protein
MCP-1	monocyte chemotactic protein-1
MCP-2	monocyte chemotactic protein-2
MMPs	matrix metalloproteinases
NMR	nuclear magnetic resonance
NO	nitric oxide
O_2^-	superoxide anion
PAI-1	plasminogen activator inhibitor type 1
PBMCs	peripheral blood mononuclear cells
phLDVP	4-((2-methylphenyl) aminocarnonyl) aminophenyl) acetyl-LDVP

PMA	phorbol 12-myristate 13-acetate
PMNs	polymorphonuclear leukocytes
RBCs	red blood cells
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
TAMs	tumor-associated macrophages
TCR	T cell antigen receptor
TGF-β	transforming growth factor-β
Th1	T helper 1 lymphocyte
Th2	T helper 2 lymphocyte
TNF-α	tumor necrosis factor-α
Treg	regulatory T cells
TSPs	thrombospondins
TSP1-	thrombospondin-1
TSRs	thrombospondin type 1 repeats
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial cell growth factor
VSMC	vascular smooth muscle cells
VWC	von Willebrand Factor type C domain

REGULATION OF INNATE IMMUNE RESPONSES BY THE EXTRACELLULAR MATRIX PROTEIN THROMBOSPONDIN-1

I. INTRODUCCIÓN

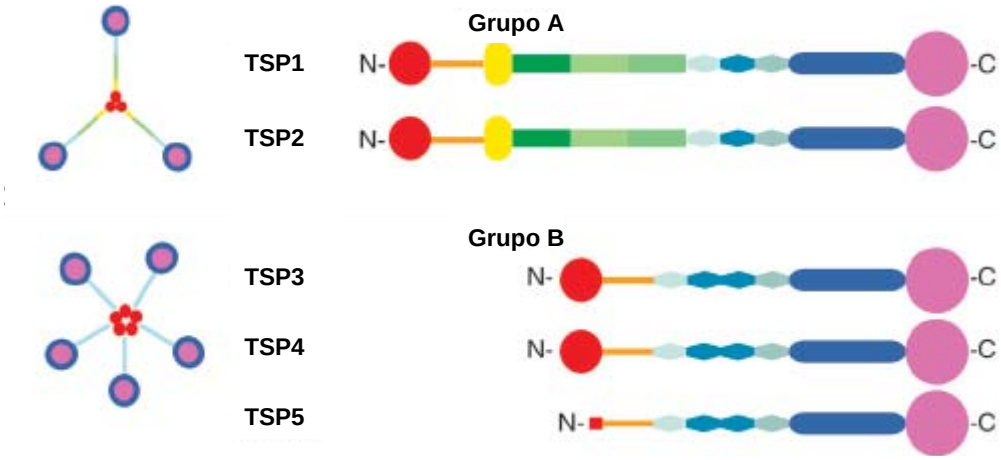
A.INTRODUCCIÓN

1. Las Trombospondinas

Las trombospondinas (TSPs) son glicoproteínas que se secretan a la matriz extracelular. Poseen una estructura de multidominios con sitios de unión a calcio (Ca^{2+}). Desde un punto de vista evolutivo, pertenecen a una familia de proteínas ancestrales, que están presentes tanto en protóstomos (e.g. *Drosophila melanogaster*, *Anopheles gambiae*) como en deuteróstomos (e.g. vertebrados). Cinco de sus miembros están codificadas en el genoma humano (TSP1 a TSP5) y cuatro de estas cinco TSPs han sido identificadas en otros vertebrados (e.g. ratón, pollo, *Xenopus laevis*, *Danio rerio*) (1).

Se puede hablar de dos subclases principales de TSPs, -A y -B atendiendo a su ensamblaje en forma de trímeros o pentámeros, respectivamente (2). La subclase-A (TSP1 y TSP2) forma trímeros de subunidades compuestas por: dominio N-terminal, un dominio de oligomerización, un dominio con el factor de von Willebrand de tipo *C* (VWC), tres dominios de repetición de tipo 1 (TSRs) o motivos de tipo properdina, un dominio de identidad que contiene tres motivos de homología al factor de crecimiento epidérmico (EGF), un haz de unión de Ca^{2+} y un dominio C-terminal de tipo lectina (**Figura 1**) (3). La subclase-B (TSP3-5 y TSPs de artrópodos) forma pentámeros, carece del dominio VWC y de los dominios TSRs, aunque tiene motivos adicionales de tipo EGF (**Figura 1**) (3). A pesar de estas diferencias, todas las TSPs comparten un dominio de identidad C-terminal muy conservado (53-82% de identidad en todos los miembros de la familia) (1).

Figura 1. Estructura modular de los miembros de la familia de Trombospondinas
*(*Adaptado de *Carlson, C.B., et al., 2008)*



Código de colores: dominio N-terminal (rojo), dominio de oligomerización (naranja), dominio con factor de von Willebrand de tipo C (VWC) (amarillo), tres dominios de repetición de tipo 1 (TSRs) o motivos de tipo properdina (verde), tres motivos de homología al factor de crecimiento epidérmico (EGF) (verde-azulado, con sitios conocidos o no de unión al calcio mas oscuros), un haz de unión de calcio (azul) y un dominio globular C-terminal de tipo lectina (morado) *(Carlson, C.B., et al., 2008)*.

Las TSPs se expresan en vertebrados durante el desarrollo embrionario y en tejidos de adulto con un patrón de expresión espacio-temporal específico (**Tabla 1**).

Tabla 1. Pricipales sitios de expresión de las Trombospondinas
*(*Adaptado de *Adams, J.C., et al., 2001)*

	Principales sitios de expresión *		
Proteína	**Embrión**	**Adulto**	**References**
TSP1	Generalizada, especialmente corazón, pulmón, epitelio intestinal, músculo esquelético (r), SNC, cartílago (p)	α-gránulos de plaquetas, endotelio activado, monocitos (h, r), endotelio de la córnea (b, h), epitelio del cristalino (b), membrana epidérmica basal, membranas epiretinales, microfibrillas arteriales (h)	Corless et al. 1992; Hiscott et al. 1997; Iruela-Arispe et al. 1993; O'Shea et al. 1988; Tooney et al. 1998; Tucker et al. 1997; Urry et al. 1998; Wight et al. 1985; Hiscott, et al. 1992; Fauvel-Lafeve, et al. 1996
TSP2	Cartílago (p, h, r), tendón (p), glándula adrenal, músculo esquelético, riñón (r)	Corteza adrenal (b, r), células de Leydig y de Purkinje, células estromales de médula (m), cartílago, tejido conectivo (h)	Adolph et al. 1999; Danik et al. 1999; Iruela-Arispe et al. 1993; Kyriakides et al. 1998; Tooney et al. 1998; Tucker et al. 1993; Bornstein et al. 2004
TSP3	SNC, médula espinal, pulmón, hueso (p, r), riñón (h), notocorda (x)	Endocrino, riñón, útero, músculo (h), SNC, intestino, pulmón (r)	Adolph et al. 1999; Iruela-Arispe et al. 1993; Tucker et al. 1997; Urry et al. 1998; Qabar et al. 1994
TSP4	Osteogénesis temprana, córnea (p), somites, músculo esquelético (x), retina en desarrollo (h)	Corazón, músculo esquelético, matriz extracelular del SNC, ojo (h), unión neuromuscular (h, r), tendón (h, b), cerebelo, retina (r)	Arber et al. 1995; Hauser et al. 1995; Lawler et al. 1993;Tucker et al. 1995; Urry et al. 1998; Stenina et al. 2003; Dunkle et al; 2007
TSP5/ COMP	Cartílago de articulaciones (r, c)	Cartílago de articulaciones, tendón, tejido sinovial (c, b, r, h), testículo (r), arterias (h)	DiCesare et al. 1994,1995; Franzen et al. 1987; Kipnes et al. 2000; Riessen et al. 2001; Hedbom et al. 1992; Sodersten et al. 2005

(*): b=bovino, p=pollo, h=humano, r=ratón, c=cerdo, x=*Xenopus*

En el adulto la mayor parte de los tejidos expresan al menos un tipo de TSP, no obstante, los distintos miembros de esta familia a menudo se expresan en poblaciones celulares diferentes dentro de un mismo tejido. Así TSP1 procede mayoritariamente de los gránulos-α de las plaquetas; aunque también se acumula en lesiones ateroscleróticas, y en tejido sinovial reumático. Además la TSP1 junto con la TSP2 se expresan de forma abundante en fibroblastos del estroma de tumores y en células endoteliales durante la progresión tumoral (4), quizás como mecanismo de defensa del propio organismo contra el tumor (5). También se expresan en procesos de renovación de tejidos como son la cicatrización de heridas en piel, músculo, y sistema nervioso central procedente de macrófagos o células de microglia, fibroblastos, y células endoteliales. Esto explicaría que los ratones con doble deficiencia en TSP1/TSP2 poseen una cicatrización anómala y persistencia del proceso inflamatorio (6). La TSP3 se expresa abundantemente en osteosarcoma metastásico (7). La TSP4 se expresa de forma diferente en carcinoma lobular invasivo/cáncer invasivo ductal (8). Se ha demostrado que los TSRs de TSP1 y TSP2, tienen cierto impacto en su actividad anti-angiogénica, por lo tanto, no cabría esperar que TSP3, 4 y 5, que carecen de TSRs, inhiban angiogénesis en el tumor.

La transcripción de la TSP4 está también regulada de forma positiva en el músculo esquelético de pacientes con distrofia muscular de Duchenne y con deficiencia en α-sarcoglicano (9). La TSP5 o COMP (*cartilage oligomeric matrix protein*) y fragmentos de la misma están presentes en cartílago dañado, suero y fluído procedente de líquido sinovial asociado con osteoartritis, lesiones en articulaciones y en procesos degenerativos del cartílago (10, 11).

Aunque hay muchos trabajos que describen las propiedades de los miembros de esta familia de TSPs, los estudios han centrado más su atención en la TSP1 y TSP2, existiendo estudios detallados sobre su síntesis y degradación. La expresión de la TSP1 está regulada a nivel transcripcional a través de múltiples sitios de unión en la región 5' del gen de la TSP1 en respuesta a numerosos estímulos extracelulares (12-14). El control post-transcripcional de la estabilidad del mRNA también está implicado en la regulación del gen (14, 15). Además, se han descrito mecanismos epigenéticos como la hipermetilación de islas CpG en torno al sitio de inicio de transcripción que están implicados en el silenciamiento de la TSP1 en algunos tipos de cáncer (16, 17).

Otro de los factores epigenéticos que regula directa e indirectamente la expresión de TSP1 es la hipoxia (18-21).

Las TSPs pueden permanecer retenidas en la matriz extracelular (ECM), aunque también pueden ser degradadas tanto en el exterior como en el interior celular. Por ejemplo, durante los procesos de fibrinólisis y resolución de coágulos la TSP1 es sustrato de degradación por la trombina y el Factor XVIIIa. TSP1 también es sustrato de catepsinas y elastasas producidas por leucocitos y neutrófilos durante la respuesta inflamatoria (22). La TSP5 es sustrato de metaloproteinasas (MMPs) como la MMP19 y MMP20 y también de otras proteasas como las ADAMTS (*membrane proteins containing a disintegrin and a metalloproteinase with TSPs motifs*) (23, 24). La TSP1 y TSP2 también están sujetas a endocitosis y posterior degradación lisosomal en el interior celular. El proceso de endocitosis es rápido y está mediado por interacciones del dominio N-terminal de la TSP con proteoglicanos tipo heparán sulfato (HSPG) y proteínas relacionados con el receptor de lipoproteínas de baja densidad (LRP), formando un complejo con la calreticulina (25). Las TSPs también pueden permanecer retenidas en la matriz extracelular, y en el caso de la TSP1 el mecanismo de retención requiere del dominio C-terminal en su forma trimérica, del motivo RGD, y de un nuevo motivo en el dominio L-lectina (26).

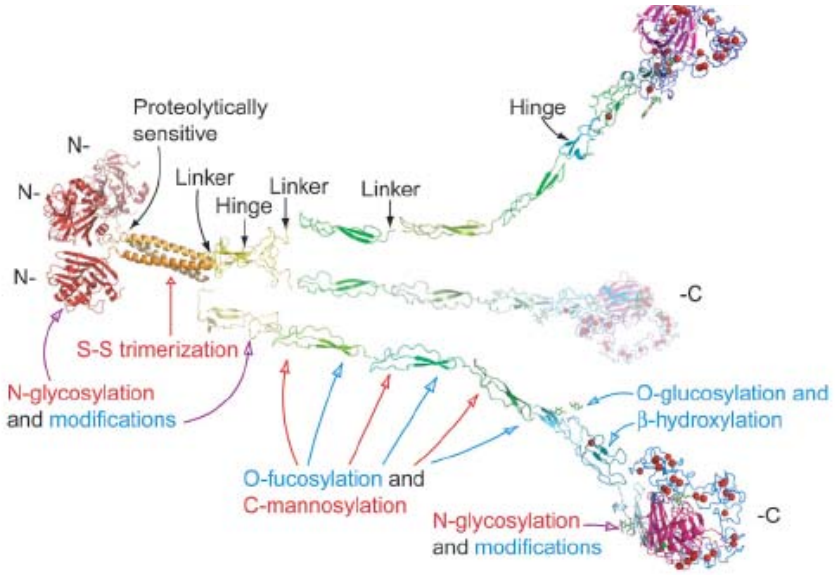
1.1. La Trombospondina-1

La TSP1 se describió en 1971 por Baenziger et al. (27), siendo el primer miembro de esta familia descrito y además el más estudiado. El nombre de esta glicoproteína viene de "***T**hrombin **S**timulated **P**latelets*", ya que fue identificada como una proteína liberada por los gránulos-α de las plaquetas en respuesta a la estimulación con trombina (27, 28).

1.1.1.Organización estructural

La compleja naturaleza de las TSPs hace imposible resolver la estructura de la molécula completa por resonancia magnética nuclear (NMR) y por cristalografía de rayos-X. El problema se ha solucionado estudiando sus fragmentos recombinantes. Estas estructuras proporcionan un modelo de trabajo para TSP1 y TSP2 en presencia de Ca^{2+} (**Figura 2**) (3).

Figura 2. Modelo de la estructura completa de la Trombospondina-1 y la Trombospondina-2
(Adaptado de *Carlson, C.B., et al., 2008)*



Código de colores: dominio N-terminal (rojo), dominio de oligomerización (naranja), dominio con factor de von Willebrand de tipo C (VWC) (amarillo), tres dominios de repetición de tipo 1 (TSRs) o motivos de tipo properdina (verde), tres motivos de homología al factor de crecimiento epidérmico (EGF) (verde-azulado, con sitios conocidos o no de unión al calcio mas oscuros), un haz de unión de calcio (azul) y un dominio globular C-terminal de tipo lectina (morado), iones de calcio (esferas rojas), azucares del dominio de identidad (palitos *Corey-Pauling-Kultin* (CPK) verdes), sitios posibles de flexibilidad estructural (negro), sitios de modificaciones co-traduccionales (rojo) y modificaciones post-traduccionales (azul) *(Carlson, C.B., et al., 2008)*.

1.1.1. A. Dominio N-terminal

Mediante búsquedas realizadas usando algoritmos basados en la homología de estructura, se ha emplazado el dominio N-terminal de TSP1 en la superfamilia de lectinas de tipo concanavalina A/glucanasas (29).

La estructura globular en β-sandwich tiene 13 hebras-β antiparalelas y 6 hélices-α, siendo la hélice α3 la más larga y cruza por encima de las dos láminas-β. Además existe una hélice-α adicional localizada cerca del extremo C-terminal del dominio. El residuo Cys214 contenido en esta hélice, forma un puente disulfuro con el residuo Cys153 ubicado en el bucle β11-β12. Este puente disulfuro aproxima el extremo C-terminal al resto del dominio N-terminal de la TSP1 (**Figura 3**) (3).

Figura 3. Estructura del dominio N-terminal de la Trombospondina-1 cristalizado
(Carlson, C.B., et al., 2008)

La estructura y los sitios de unión están coloreados de la siguiente manera: sitio de unión de heparina (palitos CPK azules), sitios de unión de fibrinógeno y α4β1 (verde), sitio de unión de calreticulina (naranja), bucle β13- β14 cubriendo la hendidura donde potencialmente se unen hidratos de carbono (amarillo), puente disulfuro (palitos CPK) *(Carlson, C.B., et al., 2008)*.

1.1.1. B. Dominio trimérico de oligomerización

Aunque la estructura del dominio de oligomerización en forma trimérica no se ha resuelto, sin embargo la estructura del dominio de oligomerización trimérico de matrilina-1 (*chicken cartilage matrix protein*), proporciona información acerca de la estructura de este dominio de TSP1 y TSP2 (30). El interior de este dominio es hidrofóbico y puede ser rígido. La superficie del dominio es hidrofílica y relativamente desordenada. El punto de unión entre el módulo N-terminal y el dominio de oligomerización (25 residuos), y el punto de unión entre el dominio de oligomerización y el módulo VWC (11-17 residuos) son sitios flexibles y sensibles a la actividad de proteasas (31).

1.1.1. C. Dominio con el factor de von Willebrand de tipo C

TSP1 y TSP2 contienen un único módulo vWC, conocido también como repetición de tipo cordina rica en cisteinas (*chordin-like cysteine-rich repeat*) o módulo N-terminal de pro-colágeno fibrilar (*N-terminal fibrillar pro-collagen module*).

La estructura atómica de este módulo no se ha resuelto, aunque se tiene información del módulo vWC de TSP1 gracias a estudios biofísicos de baja resolución (32). Este dominio contiene 10 residuos de cisteina, con un grado de conservación relativamente bajo, a excepción de las cisteinas segunda y octava (33). Su análisis mediante espectrometría de masas revela que se trata de un modulo glicosilado en el que todos los residuos de cisteina están implicados en la formación de puentes disulfuro que estabilizan la proteína.

1.1.1. D. Dominios de repetición o TSRs.

TSP1 y TSP2 contienen tres TSRs en tándem. Estos TSRs, de unos 60 amino ácidos de longitud, están evolutivamente conservados y contienen unos 12 residuos invariables que consisten en 6 cisteinas, 2 residuos de arginina y 2 ó 3 residuos de triptófano separados a su vez por 2 ó 4 amino ácidos (34, 35).

En estos dominios se han descrito dos tipos de glicosilación poco comunes: La C-manosilación de los residuos de triptófano, co-traduccional y mediada por la enzima dolichol-P-manosa. Esta tiene lugar en la secuencia WXXW del TSR (36); y la O-fucosilación, modificación post-traduccional mediada por la enzima O-fucosiltransferasa-2, que tiene lugar en la secuencia CSX(S/T)C del TSR en residuos de serina o treonina (37) y que es crítica en el proceso de maduración y secreción de la proteína (37, 38).

1.1.1. E. Dominio de identidad

Esta porción de la proteína incluye los motivos en tándem de tipo EGF, 13 sitios de unión al Ca^{2+} que constituyen el haz de unión de Ca^{2+} y un módulo C-terminal de tipo lectina. En la familia de las TSPs la diferencia más grande en estos dominios de identidad se encuentra en el número de motivos de tipo EGF (3 en la TSP1 y TSP2) (1). EGF1 contiene un sitio que está modificado por la enzima glucosa-xilosa disacaridasa en TSP1 bovina. EGF2 une un único Ca^{2+} en la interfase entre EGF1 y EGF2 y probablemente estabiliza las interacciones entre los módulos.

EGF3 interacciona con EGF2, con las porciones N- y C-terminal del haz de unión de Ca^{2+} y con el módulo de tipo lectina. El haz de unión de Ca^{2+} forma una estructura estable, dependiente de Ca^{2+} e independiente del resto del dominio de identidad y transmite los cambios alostéricos inducidos por iones de Ca^{2+} a otras partes de TSP (3, 39). Este dominio consta de 13 motivos ricos en aspartato que forman conformaciones globulares individuales conectadas y estabilizadas por puentes disulfuro. Como media, cada motivo une dos iones Ca^{2+}, y la unión de los mismos causa un cambio conformacional en la proteína que ayuda a mantener los dominios de identidad de la TSP en una conformación más compacta (40). En cuanto a su estructura, el módulo de tipo lectina está compuesto por una hélice-α y dos láminas-β, cada una de las cuales continen siete hebras-β. De las dos láminas-β, una forma una curva convexa en la superficie de la proteína y la otra está más doblada hacia el interior de la proteína. La hélice-α se sitúa en la hendidura formada por la segunda lámina-β. La búsqueda de homologías usando algoritmos basados en estructura, sitúan este módulo dentro de la familia con dominios de lectina de tipo-L

1.1.2.Principales sitios de expresión

La TSP1 se expresa en diversos tejidos en el adulto y también durante el desarrollo, siempre con un patrón de expresión complejo. No obstante, los principales cambios ocurren durante condiciones fisiopatológicas. Por ejemplo, en respuesta a daños tisulares se liberan de forma rápida y transitoria concentraciones elevadas de TSP1 (6, 41). La TSP1 se expresa durante la cicatrización de lesiones en la piel, el hueso (42), el músculo, el nervio (43, 44), en lesiones ateroscleróticas e hiperplasia de la íntima (45, 46). La TSP1 se expresa durante la coagulación con una distribución espacio-temporal característica (47). Además la TSP1 está elevada en algunas enfermedades coronarias crónicas (48). Se expresa también en los fibroblastos de la córnea e iris en el síndrome de pseudoexfoliación (49), en el sinovium reumático (50) y en los fibroblastos del estroma de tumores y células endoteliales durante estadíos tempranos de la progresión tumoral (4), quizás como un mecanismo de defensa del propio organismo contra el tumor (5).

No obstante, con frecuencia la expresión de la TSP1 se pierde durante la progresión del tumor. Esto se debe en parte a la regulación negativa mediada por oncogenes tales como Ras, Myc, y otros factores como Id1, y también en parte a la ausencia de regulación positiva por parte de genes supresores de tumores como p53 (51-54).

1.1.3.Conexiones moleculares

La TSP1 se une a glicoproteínas transmembrana que sirven de receptores de señalización. Así se ha visto que se une a integrinas activadas de tipo α3β1, α6β1, α9β1, y α4β1 (3, 55-57). Los sitios de unión para estos receptores han sido mapeados usando péptidos sintéticos. El dominio N-terminal de TSP1 contiene sitios consenso BBXB de alta afinidad de unión a heparina/HSPG. Los TSRs contribuyen a la unión de heparina con múltiples sitios de baja afinidad (58, 59), estos también median interacciones de TSP1 con CD36 (60, 61) y con integrinas β1 (62, 63).

Además la TSP1 también se une a otras moléculas extracelulares tales como componentes de matriz, proteasas, citoquinas y factores de crecimiento. Entre estos encontramos la decorina (64), fibrinógeno (29), calreticulina (3), LRP (65), fibronectina (66), la metaloproteinas de tipo 2 (MMP-2) (67), y TGF-β1 (68). El motivo WSHWSPW en el segundo TSR se une a TGF-β1 latente. Como resultado, TGF-β1 maduro y activo se libera por efecto de la interacción molecular con el motivo KRFK en el primer TSR de la TSP1 (68). Esta interacción con TGF-β1 es relevante para la función homeostática de TSP1. El haz de unión de Ca^{2+} en TSP1 contiene también sitios de unión para integrinas de tipo αvβ3 y αIIbβ3 (69), y otras proteínas como la catepsina G y la elastasa de neutrófilos (70). El módulo C-terminal de TSP1 de tipo lectina contiene el sitio de unión para CD47 (71).

A menudo, la unión de la TSP1 a otras moléculas modula la actividad de las mismas. Por ejemplo, la unión de TSP1 a catepsina G y elastasa de neutrófilos o MMP-2 disminuye la actividad catalítica de estas enzimas. Este proceso puede ser importante para la fibrilogénesis del colágeno y para la regulación de la angiogénesis.

La expresión diferencial y el grado de activación de los receptores celulares en membrana para la TSP1, tales como integrinas, CD36, CD47, LRP, proteoglicanos y sulfátidas, puede determinar que haya respuestas específicas a TSP1 dependientes de cada tipo celular (72-74).

1.1.4.Funciones de la Trombospondina-1

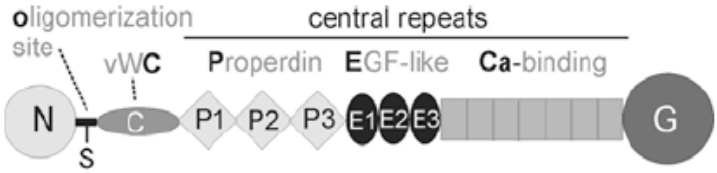
Tanto el estudio de ratones deficientes para el gen de la TSP1 (75), como la existencia de enfermedades genéticas humanas relaccionadas con la ausencia de la TSP1 ponen en evidencia funciones específicas de la proteína *in vivo*.

Los ratones genéticamente nulos para la TSP1 son fértiles y se desarrollan con normalidad. No obstante, la viabilidad del embrión está disminuida, y los fenotipos incluyen pneumonía (a partir de un mes de edad), lordosis espinal leve y un aumento en el número de monocitos circulantes.

La pneumonía e inflamación anormal en los pulmones y el páncreas propia de los ratones nulos para la TSP1, presenta muchas similitudes fenotípicas con los ratones nulos para TGF-β1 (76) y son una consecuencia de la falta específica de activación de TGF-β1 en estos tejidos. Aunque la TSP1 no es el único activador de TGF-β1, parece tener un papel dominante en la activación del mismo en estos dos tejidos, requisito necesario tanto para una homeostasis epitelial normal como para el control de las respuestas inmunes (77). Además de estos defectos menores, se han descrito funciones adicionales de la TSP1 al al someter a estos ratones a determinados tipos de estrés fisiológico (78).

Los estudios funcionales han puesto de manifiesto que la TSP1 participa además en procesos celulares tales como, adhesión, organización del citoesqueleto, migración, crecimiento de neuritas, agregación celular, proliferación, apoptosis, diferenciación y regulación transcripcional (**Figura 4**).

Figura 4. Diagrama esquemático de las interacciones moleculares y las funciones de las distintas subunidades de la Trombospondina-1



Interacciones moleculares	Calreticulina Integrinas α4β1, α3β1, α6β1, α9β1 Fibrinógeno HSPG/Heparina Decorina LRP	CD36 Fibronectina TGF-β MMP-2 Integrinas β1 HSPG	Integrinas αvβ3, αIIbβ3 Catepsina G Elastasa	CD47
Funciones	Adhesión celular, migración, agregación plaquetaria Endocitosis Desensamblaje de adhesiones focales Regulación de la proliferación	Adhesión celular Interacciones con la matriz Crecimiento de neuritas Regulación de células endoteliales (inhibición de la proliferación y angiogénesis, inducción de apoptosis) Agregación plaquetaria (inhibición del retraso en la agregación en respuesta a NO) Regulación de la homeostasis epitelial, respuesta inmune (páncreas, pulmón)	Adhesión celular Inhibición de proteasas	Adhesión celular, migración, agregación plaquetaria Proliferación de células de musculatura lisa Activación de células T Inhibición de la señalización por NO

La TSP1 sirve de sustrato para la adhesión de muchos tipos celulares. Después de la adhesión, las células pueden permanecer redondas y estáticas o extenderse y hacerse móviles. Los fenómenos de extensión celular y migración sobre sustratos de TSP1 se asocian con procesos de organización del citoesqueleto cortical que conllevan la formación de largos lamelipodios (79). Los efectos de la TSP1 en el citoesqueleto de actina son diversos y dependen del tipo celular. Las células mesangiales intraglomerulares, monocitos, neutrófilos, células de músculo liso, mioblastos esqueléticos y células de carcinoma, son algunos ejemplos de células que se extienden y adquieren movilidad en presencia de TSP1. Los efectos de la TSP1 en la movilidad de células vasculares de músculo liso (VSMC) son relevantes en el contexto de lesiones ateroscleróticas y vasculares. Los fenómenos de quimiotaxis en respuesta a TSP1 dependen de los receptores CD47, integrinas de tipo αv (80), e integrinas de tipo β1 (81). El tratamiento de arterias dañadas con anticuerpos contra el sitio de unión a CD47 en TSP1 reduce el engrosamiento de la neoíntima (82). Además, se han descrito polimorfismos en TSP1, TSP2 y TSP4 que aumentan el riesgo de sufrir enfermedad coronaria prematura, probablemente debido a un incremento de la actividad pro-aterogénica o pro-trombótica (83). No obstante, un análisis reciente de poblaciones más grandes ha puesto en duda la validez de esta asociación (84).

Por otro lado, la TSP1 es un fuerte antagonista de la relajación de VSMC promovida por óxido nítrico (NO), sugiriendo que la TSP1 juega un papel importante en el control de la perfusión de los tejidos (85). Tras un accidente isquémico agudo, ratones nulos para TSP1 y CD47 presentan un mayor índice de supervivencia del tejido (86, 87). Además la TSP1 también promueve fenómenos de adhesión y agregación célula-célula. Así, participa en la adhesión de eritrocitos (RBCs) infectados con el parásito de la malaria al endotelio vascular (88). La TSP1 se une directamente a los eritrocitos en forma de hoz (*Sickle* RBCs) (89) y participa en su adhesión a células endoteliales *in vitro* (90), contribuyendo a la patogénesis de oclusión vascular característica de la enfermedad de *Sickle*.

La TSP1 también juega un papel importante en la fisiología plaquetaria (agregación plaquetaria y formación del coágulo de fibrina). Así regula la fisiología plaquetaria mediante la prevención de la degradación del factor de von Willebrand por la proteasa plamática ADAMTS-13. Esto se pone de manifiesto en ratones nulos para TSP1 que presentan una adhesión defectuosa de los trombos (91).

Un segundo mecanismo a través del cual la TSP1 modula la fisiología plaquetaria consite en la inhibición de la actividad fisiológica anti-trombótica del NO (92). El NO juega un papel fundamental en la fisiología plaquetaria, al limitar su agregación (93). Tanto la proteína completa como fragmentos recombinantes de los TSRs y del dominio C-terminal, inhiben el retraso en la agregación promovido por el NO mediante un mecanismo que implica la inhibición de múltiples pasos en la cascada de señalización del NO/cGMP (92).

Los efectos de la TSP1 en la proliferación celular son también específicos del tipo celular. Por ejemplo, la TSP1 coopera con factores de crecimiento tales como EGF o PDGF para promover la proliferación de SMC (94). En el caso de células endoteliales, TSP1 inhibe la proliferación e induce activamente la apoptosis (95-97). CD36 se considera un receptor crítico en la actividad anti-angiogénica de la TSP1, dado que en un modelo experimental de angiogénesis en la córnea de ratones nulos para CD36 no se observa actividad anti-angiogénica (98).

No obstante, la cornea avascular es un tejido especializado que se mantiene gracias a la sobre-expresión del receptor-1 del factor de crecimiento de endotelio vascular (VEGF) en forma soluble (99). Este ambiente es permisivo para la angiogénesis estimulada por el factor de crecimiento de fibroblastos (FGF)-2 que es independiente del NO, pero no para la angiogénesis estimulada por VEGF que está mediada por NO. Al contrario que en el modelo experimental de angiogénesis en la córnea, el crecimiento de vasos en explantes musculares procedentes de ratones nulos para CD36 permanece sensible a la inhibición por TSP1 (87). Esto indica que existen otros receptores de la TSP1 que estarían mediando esta actividad (100). Los motivos peptídicos adyacentes al sitio de unión de CD36 también inhiben angiogénesis. Estos motivos peptídicos podrían fortalecer la unión de TSP1 a CD36 o tener efectos indirectos tales como el secuestro de factores pro-angiogénicos (97, 101, 102). Aunque la interacción de TSP1 con CD36 juega un papel importante en la actividad anti-angiogénica de la TSP1, también se han identificado mecanismos independientes de CD36.

Los TSRs pueden unir integrinas de tipo β1 (62) y estimular la migración de células endoteliales (103). CD47 también se ha relacionado con un incremento en la apoptosis de células endoteliales (104). Y por último, péptidos del dominio de identidad de TSP1 de unión a CD47 también inhiben angiogénesis en la córnea de ratón (105). El crecimiento de vasos en explantes de músculo procedentes de ratones nulos para CD47 no es susceptible de inhibición por TSP1, indicando que CD47 es necesario para la respuesta anti-angiogénica de TSP1 en este ensayo (106). Además, TSP1 y dominios recombinantes de esta no inhiben la vía de señalización del NO en células endoteliales procedentes de ratones nulos para TSP1. Para terminar, la angiogénesis en injertos de piel de espesor total está potenciada en ratones nulos para TSP1 y CD47 pero no en los ratones nulos para CD36 (107). Por lo tanto CD47 y no CD36, es el receptor de TSP1 esencial para la inhibición de angiogénesis en isquemia.

Además, TSP1 inhibe angiogénesis a través de sus efectos en la biodisponibilidad de VEGF. La TSP1 bloquea la liberación de VEGF desde ECM inhibiendo la activación de MMP-9 (108). Esta también se une directamente a VEGF favoreciendo su incorporación y degradación celular (109).

La TSP1 activa TGF-β latente en el entorno del tumor (110) y esta actividad también podría contribuir al letargo del tumor. A menudo, TSP1 está regulada negativamente durante la progresión tumoral (111) y esta pérdida se asocia a alteraciones en oncogenes específicos y genes supresores de tumores (p53, Myc, Ras) que controlan la expresión de TSP1 (51, 53).

1.1.4.A. Regulación de las Respuestas Inmunes

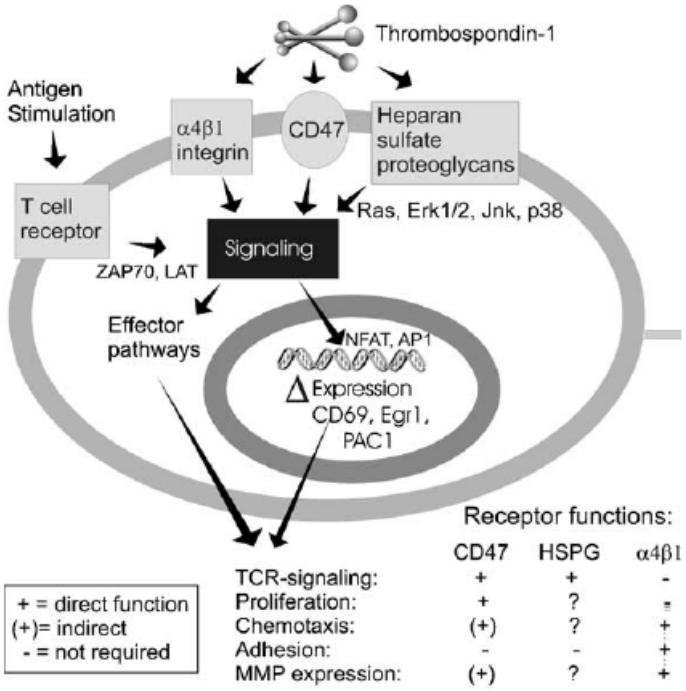
Los ratones nulos para TSP1 manifiestan un fenotipo que incluye infiltrados inflamatorios pulmonares agudos y crónicos, y un número elevado de leucocitos en circulación (77), lo cual sugiriere que la TSP1 tiene también un papel anti-inflamatorio. Como se menciona previamente en la página 19, la pneumonía e inflamación anormal en los pulmones y el páncreas de estos ratones puede ser una consecuencia de la falta específica de activación de TGF-β1 latente en estos tejidos, requerida para la homeostasis epitelial normal y el control de la respuesta inmune (77).

Una de las principales funciones de TGF-β es mantener la tolerancia de las células T a autoantígenos a través de la inhibición directa de la diferenciación de linfocitos T cooperadores (Th1 y Th2), y linfocitos T citotóxicos (CTL) y el mantenimiento de células T reguladoras (Treg) (112). La proliferación de células asesinas naturales humanas también está regulada por la activación de TGF-β1 latente mediada por TSP1 (113). En un principio, TGF-β1 está enmascarado por la asociación no covalente con un dímero de su propéptido N-terminal, denominado proteína asociada a latencia (LAP) (114, 115). Los TSRs centrales de la TSP1 interaccionan con la región N-terminal de LAP formando un complejo de tres moléculas (116). En este complejo tiene lugar un cambio conformacional que hace que TGF-β1 esté accesible a su receptor (77). Además la TSP1 parece tener un papel dominante en la activación de TGF-β1 latente especialmente en los pulmones y el páncreas.

La TSP1 también regula de forma directa la fisiología de las células T. Estudios basados en el uso *de microarrays* de cDNA indican que TSP1 es fundamentalmente un inhibidor de la transducción de señales vía TCR (117), aunque algunos péptidos muestran actividades diferentes a las de la proteína completa.

Los TSRs de TSP1 se unen a HSPG en células T y estimulan la activación del oncogén Ras inducida por CD3 así como la fosforilación en tirosinas de las tres MAP kinasas Erk1/2, JNK y p38 (118). Para analizar el papel de los receptores de TSP1 en estas funciones se usaron líneas celulares de células T con mutaciones somáticas que las hacen deficientes en integrinas de tipo β1 o en CD47 (119). Estos estudios indicaban que la integrina α4β1 es necesaria y suficiente para estimular la adhesión de células T, y también para la estimulación de quimiotaxis y expresión génica de MMPs mediada por TSP1 y TSP2 (119). Estos autores ya habían visto previamente que el receptor CD47 juega un papel significativo en la inhibición de la señalización vía TCR y en la proliferación de células T (117), así como en la inhibición de la diferenciación de células T *naive* hacia efectores Th1 (120, 121) (**Figura 5**).

Figura 5. Transducción de señales por *Trombospondina-1* en linfocitos T
(Kuznetsova, S.A., et al., 2004)



Los ratones nulos para CD47 presentan defectos en la movilidad de los leucocitos y en las respuestas frente a bacterias patógenas (122). Este fenotipo está en consonancia con evidencias de que CD47 controla la activación de α4β1 (123), requerida para la diapédesis de leucocitos en respuesta a algunas señales inflamatorias. La TSP1 también puede regular las funciones de las células T indirectamente, a través de sus efectos en células presentadoras de antígeno (124, 125).

1.1.4.B. Regulación de las Respuestas Inmunes Innatas

Las proteínas de ECM liberadas en sitios de inflamación o lesión pueden modular los procesos de fagocitosis, adhesión, movilidad, generación de oxidantes, etc mediados por leucocitos polimorfonucleares (PMNs). Aunque el mayor reservorio de la TSP1 está en los gránulos α de las plaquetas también la secretan, aunque en menor medida, otros tipos celulares tales como, monocitos, macrófagos y células dentríticas (DCs). en las cuales desempeña diversas funciones relevantes en la fisiología de estas células fagocíticas. La TSP1 procedente de monocitos apoptóticos y su HBD promueven un estado tolerogénico y fagocítico en DCs inmaduras (124). En eosinófilos y neutrófilos senescentes la TSP1 media los procesos de fagocitosis mediada por macrófagos. Esto sería un mecanismo limitante de daño asociado a la acumulación de deshechos celulares que implica la unión de la TSP1 a los receptores αvβ3 y CD36 (126, 127).

Los macrófagos median la fagocitosis de eosinófilos apoptóticos por un mecanismo independiente de la secreción de quimioquinas. Por el contrario, la fagocitosis de eosinófilos post-apoptóticos puede desencadenar respuestas pro-inflamatorias (126). El proceso de fagocitosis de neutrófilos apoptóticos por macrófagos puede ser potenciado por citoquinas proinflamatorias tales como TGF-β1, y factores estimulantes de colonias de macrófagos y granulocitos (GM-CSF), como un mecanismo de regulación del número de neutrófilos en sitios de inflamación (127). El reconocimiento y la fagocitosis de fibroblastos apoptóticos por parte de los macrófagos depende de TSP1 procedente de los fibroblastos y de CD36 (128). Además, los fagocitos semiprofesionales, tales como células mesangiales, median la fagocitosis de neutrófilos apoptóticos por un mecanismo dependiente de αvβ3/TSP1 e independiente de CD36 y de la secreción de quimioquinas (129).

La TSP1 puede regular la agregación homotípica de la línea celular monocítica humana U937 mediada por el antígeno asociado a función leucocitaria (LFA-1) y por la molécula de adhesión intracelular (ICAM-1) a través de su efecto inhibidor vía CD47 o de su efecto activador vía CD36 (130). Por otro lado, la TSP1 desempeña un papel fundamental en inflamación y aterogénesis al aumentar la adhesión de monocitos al endotelio, mecanismo que conlleva la inducción de la molécula de adhesión vascular (VCAM-1) y de la molécula de adhesión intracelular ICAM-1 (131). A concentraciones elevadas, y en solución, la TSP1 estimula la movilidad de neutrófilos humanos (132, 133) y promueven quimiotaxis y haptotaxis de monocitos humanos (134).

Además, la TSP1 puede desempeñar un papel en la diferenciación de leucocitos así como en la expresión de citoquinas. En este sentido estaría implicada en la diferenciación de la línea celular de leucemia mieloide HL-60 inducida por ácido retinoico (135). La unión a CD47 del péptido derivado de TSP1, 4N1K, durante la diferenciación de monocitos en DCs, reduce la expresión de la IL-12 y del TNF-α (136). De esta manera, la TSP1 procedente de DCs sirve de regulador negativo y contribuye a la paralización de la producción de citoquinas, resolución de la inflamación y mantenimiento del *steady state* (125, 137). Pacientes deficientes en CD36 presentan respuestas defectuosas en la activación de NF-κB inducida por la lipoproteína de baja densidad (LDL) oxidada y posterior expresión de citoquinas (138).

La TSP1 modula la expresión de IL-6 e IL-10 por parte de los monocitos (139) y la activación de TGF-β latente (77). Además, mediante su unión a neutrófilos humanos a través de los residuos F16-G33 y A784-N823 (140), potencia el *respiratory burst* inducido por citoquinas (141) y la generación de anión superóxido (O_2^-) inducida por el factor quimiotáctico *n-formyl-methionyl-leucyl-phenylalanine* (FMLP) en neutrófilos humanos a través de su dominio N-terminal (140, 142). No obstante, el mecanismo fundamental en la regulación de la generación de O_2^- no ha sido identificado.

En esta tesis se aportan evidencias de que TSP1 en solución causa un aumento significativo en la generación de O_2^- estimulada con *phorbol 12-myristate 13-acetate* (PMA) en monocitos humanos diferenciados con *interferon-γ* (INF-γ), por interacción de la región N-terminal de la TSP1 con su receptor de tipo integrina α6β1. Además hemos observado que este fenómeno requiere de Ca^{2+} extracelular como segundo mensajero en macrófagos.

1.1.4.C. Regulación de la Respuesta Inmune Innata mediada por Macrófagos en contra del tumor

Los macrófagos son células efectoras importantes en la respuesta inmune innata en contra de tumores. No obstante, los macrófagos asociados a tumores (TAMs) pueden adquirir un fenotipo citotóxico (M1) o promotor del creciemiento tumoral (M2). Por otra parte se ha visto que el proceso de diferenciación de los TAMs depende del microambiente del tejido (143). Los macrófagos se activan por la vía clásica y adquieren un fenotipo M1 en respuesta a IFN-γ sólo o en concierto con productos procedentes de microbios. También se activan por la vía alternativa y adquieren un fenotipo M2 en respuesta a la estimulación con IL-4 o IL-13, IL-10, IL-21, TGF-β, inmunocomplejos y glucocorticoides (144). En general, los macrófagos M2 están presentes en tumores establecidos y promueven la progresión del tumor (145).

La TSP1 a menudo se regula negativamente durante la progresión tumoral e inhibe el crecimiento tumoral cuando se re-expresa. Esta actividad se atribuye normalmente a la inhibición de la angiogénesis, pero los resultados presentados a continuación sugieren que debemos tener en cuenta los efectos en la inmunidad tumoral. El presente estudio pone de manifiesto la importancia de la TSP1 como modulador positivo de la inmunidad innata anti-tumoral aumentando el reclutamiento de macrófagos y estimulando la citotoxicidad tumoral mediada por especies reactivas de oxígeno (ROS).

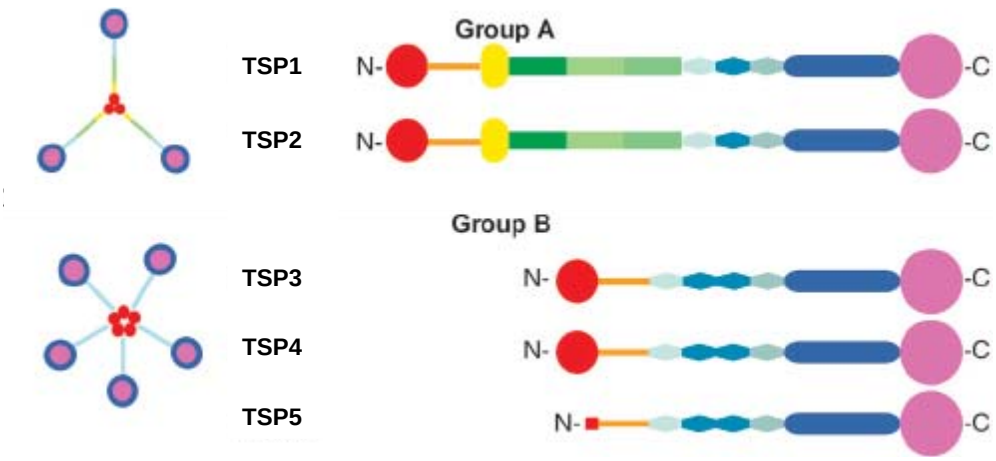
B.INTRODUCTION

1.Thrombospondins

Thrombospondins (TSPs) are an evolutionary ancient family of multidomain, calcium-binding, secreted glycoproteins, present in both protostome (e.g. *Drosophila melanogaster*, *Anopheles gambiae*) and deuterostome animals (e.g vertebrates). Five TSPs are encoded in the human genome (TSP1 through TSP5), and four to five TSPs have been identified in other vertebrates (*i.e*. mouse, chicken, *Xenopus laevis*, *Danio rerio*) (1). There are two major subclasses of TSPs, -A, and -B according to their assembly as trimers or pentamers, respectively (2).

The subclass-A (TSP1 and TSP2) form trimers of subunits that are composed of an N-terminal domain, an oligomerization domain, a von Willebrand Factor type C (VWC) domain, three properdin-like repeats or thrombospondin type 1 repeats (TSRs), and a signature domain comprising three epidermal growth factor (EGF)-like repeats, a calcium binding wire and a lectin-like C-terminal globe (**Figure 1**) (3). The subclass-B (TSP3-5 and arthropod TSPs) form pentamers, lack VWC modules and TSRs, and have additional EGF-like repeats (**Figure 1**) (3). All TSPs share a highly conserved C-terminal signature domain (53-82% identity across the whole family) (1).

Figure 1. Modular structures of Thrombospondin family members
(Modified from Carlson, C.B., et al., 2008)



Color code: N-terminal domain (red), oligomerization domain (orange), a von Willebrand Factor type C (VWC) domain (yellow), three properdin-like repeats or thrombospondin repeats (TSRs) (shades of green), three epidermal growth factor (EGF)-like repeats (shades of blue-green, with predicted or known calcium-binding repeats colored in the darkest shade), a calcium binding wire (blue), and a lectin-like C-terminal globe (purple) (Carlson, C.B., et al., 2008).

TSPs are expressed in vertebrates during embryonic development and in adult tissues with a specific spatiotemporal pattern of expression (**Table 1**).

Table 1. Major sites of expression of Thrombospondins
(Modified from Adams, J.C., et al., 2001)

	Major sites of expression *		
Protein	**Embryonic**	**Adult**	**References**
TSP1	Widespread, especially heart, lung, intestinal epithelium, skeletal muscle (m), CNS, cartilage (ch)	Platelet α-granules, activated endothelium, monocytes (h, m), corneal endothelium (b, h), lens epithelium (b), epidermal basement membrane, epiretinal membranes, arterial microfibrils (h)	Corless et al. 1992; Hiscott et al. 1997; Iruela-Arispe et al. 1993; O'Shea et al. 1988; Tooney et al. 1998; Tucker et al. 1997; Urry et al. 1998; Wight et al. 1985; Hiscott, et al. 1992; Fauvel-Lafeve, et al. 1996
TSP2	Cartilage (ch, h, m), tendon (ch), adrenal, skeletal muscle, kidney (m)	Adrenal cortex (b, m), Leydig and Purkinje cells, marrow stomal cells (m), cartilage, conective tissues (h)	Adolph et al. 1999; Danik et al. 1999; Iruela-Arispe et al. 1993; Kyriakides et al. 1998; Tooney et al. 1998; Tucker et al. 1993; Bornstein et al. 2004
TSP3	CNS, spinal cord, lung, bone (ch, m), kidney (h), notocord (x)	Endocrine, kidney, uterus, muscle (h), CNS, gut, lung (m)	Adolph et al. 1999; Iruela-Arispe et al. 1993; Tucker et al. 1997; Urry et al. 1998; Qabar et al. 1994
TSP4	Early osteogenesis, cornea (ch), somites, skeletal muscle (x), developing retina (h)	Heart, skeletal muscle, extracellular matrix of the CNS, eye (h), neuromuscular junctions (h, m), tendon (h, b), cerebellum, retina (m)	Arber et al. 1995; Hauser et al. 1995; Lawler et al. 1993;Tucker et al. 1995; Urry et al. 1998; Stenina et al. 2003; Dunkle et al; 2007
TSP5/ COMP	Articular cartilage (m, p)	Articular cartilage, tendon, synovium (p, b, m, h), testis (m), arteries (h)	DiCesare et al. 1994,1995; Franzen et al. 1987; Kipnes et al. 2000; Riessen et al. 2001; Hedbom et al. 1992; Sodersten et al. 2005

* Species: b=bovine, ch=chick, h=human, m=mouse, p=porcine, x=xenopus

Most adult tissues express at least one TSP, however, within a given tissue, individual family members are often expressed in non-overlapping cell populations. However, major changes occur during pathophysiological conditions. TSP1 is released from platelets α-granules; it accumulates in atherosclerotic lesions, and rheumatoid synovium. TSP1 and TSP2 are highly expressed by stromal fibroblasts and endothelial cells within tumors during tumor progression (4), maybe as a host anti-tumor defense mechanism (5), and during the tissue remodeling in healing wounds in skin, muscle, and the central nervous system through expression by macrophages or microglia, fibroblasts, and endothelial cells. Aberrant wound healing and prolonged persistence of inflammation is observed in double-TSP1/TSP2 null mice (6). TSP3 is highly expressed in metastatic osteosarcoma (7). TSP4 is differentially expressed in invasive lobular breast carcinoma as compared to ductal breast carcinoma (8). The TSRs of TSP1 and TSP2, have been shown to at least impact their anti-angiogenic activity. Thus, TSP3, 4 and 5, that lack TSRs, would not be expected to inhibit angiogenesis in the tumor microenvironment. TSP4 transcripts are also upregulated in the skeletal muscle of Duchene muscular dystrophy patients and α-sarcoglycan-deficient patients (9).

TSP5/COMP (cartilage oligomeric matrix protein) and COMP fragments in diseased cartilage, serum and synovial fluid have been correlated with osteoarthritis, joint injury and cartilage degradation (10, 11).

TSP1 and TSP2 are the two members of this family that have been most intensively studied in terms of protein synthesis and degradation. TSP1 gene expression is regulated at the transcriptional level through multiple binding sites within the 5' flanking region of TSP1 gene in response to multiple extracellular stimuli (12-14). A post-transcriptional control of mRNA stability is also involved in the regulation of the gene (14, 15). Moreover, epigenetic mechanisms such as hypermethylation of CpG islands around the transcription start site are also involved in the silencing of TSP1 expression in cancer (16, 17). Hypoxia may also modulate epigenetic mechanisms as well as directly or indirectly influence TSP1 expression (18-21).

Extracellular TSPs can be degraded extracellularly, intracellularly, or be retained within the extracellular matrix (ECM). During fibrinolysis and clot resolution, TSP1 is a substrate for cleavage by thrombin and Factor XVIIIa. TSP1 is also a substrate for the cathepsins and elastases released by leucocytes and neutrophils during an inflammatory response (22). TSP5 is a substrate for metalloproteinases (MMPs) 19 and 20 and ADAMTS (membrane proteins containing a disintegrin and a metalloproteinase with TSPs motifs) (23, 24). TSP1 and TSP2 are also degraded intracellularly through endocytosis and lysosomal degradation. The rapid endocytic uptake is mediated by interactions of the TSP N-terminal domain with heparan sulphate proteoglycans (HSPG) and the low-density lipoprotein (LDL) receptor-related protein (LRP), in a complex with calreticulin (25). TSPs can also undergo retention within ECM. ECM retention is a conserved property of TSPs, and the mechanism of retention with regard to TSP1 involves the trimeric form of the C-terminal domain, the RGD motif, and a novel site in the L-lectin domain (26).

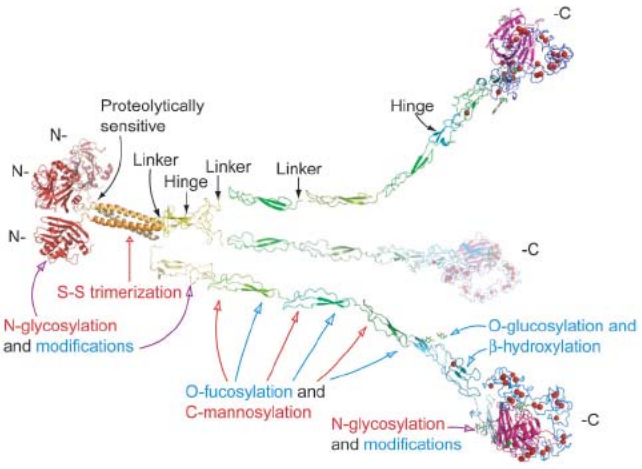
1.1.Thrombospondin-1

TSP1 is the first member of this family described by Baenziger et al. in 1971 (27), and is the one that has been most intensively studied. This ECM glycoprotein was termed TSP because it was identified as a glycoprotein released from α-granules of platelets in response to stimulation with thrombin (27, 28).

1.1.1.Structural organization

The complex nature of TSPs makes solving the structure of entire molecules impossible by nuclear magnetic resonance (NMR) and very difficult by X-ray crystallography. The problem has been solved by studying recombinant fragments of TSPs. These structures provide a working model for TSP1 and TSP2 in high calcium (Ca^{2+}) (**Figure 2**) (3).

Figure 2. Model of the complete structure of Thrombospondin-1 and Thrombospondin-2
(Modified from Carlson, C.B., et al., 2008)



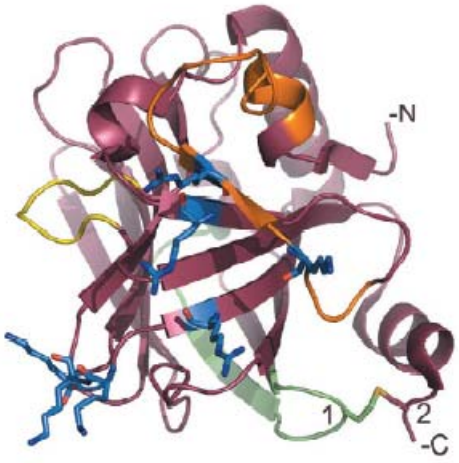
Color code: N-terminal domain (red), oligomerization domain (orange), a von Willebrand Factor type C (VWC) domain (yellow), three properdin-like repeats or thrombospondin repeats (TSRs) (shades of green), three epidermal growth factor (EGF)-like repeats (shades of blue-green, with predicted or known calcium-binding repeats colored in the darkest shade), a calcium binding wire (blue), and a lectin-like C-terminal globe (purple), calcium ions (red spheres), signature domain sugars (green Corey-Pauling-Kultin (CPK) sticks), possible sites of structural flexibility (black), sites of co-translational modifications (red), and post-translational modifications (blue) (Carlson, C.B., et al., 2008).

1.1.1. A. N-terminal domain

Homology-searching algorithms based on structure, placed the N-terminal domain of TSP1 in the concanavalin A-like lectins/glucanases superfamily (29). The globular β-sandwich structure has 13 antiparallel β-strands, and 6 α-helices. Helix α3 is the largest and crosses over the top of the two β-sheets.

There is an additional α-helix located near the C-terminal end of the domain. This helix contains Cys214, which forms a disulfide bond with Cys153 found in the β11-β12 loop. This disulfide bond brings the C terminus into a close proximity to the rest of the N-terminal domain of TSP1 (**Figure 3**) (3).

Figure 3. Crystal structure of the N-terminal domain of Thrombospondin-1
(Carlson, C.B., et al., 2008)



Crystal structure and binding sites are are colored as follows: heparin-binding region (blue CPK sticks), fibrinogen and a4b1 binding sites (green), calreticulin binding site (orange), b13-b14 loop covering potential carbohydrate-binding cleft (yellow), disulfide bond (CPK sticks).

1.1.1. B. Trimeric oligomerization domain

The structure of the trimeric oligomerization domain has not been solved. However, the structure of the trimeric oligomerization domain of matrilin-1 (chicken cartilage matrix protein), provides insight into the structure of this domain of TSP1 and TSP2 (30).

The core of the domain is hydrophobic and may be rigid. The surface of the domain is hydrophilic and relatively disordered. The linkage between the N-terminal module and the oligomerization domain (25 residues), and the linkage between the oligomerization domain and the VWC module (11-17 residues) are sites of flexibility and protease sensitivity (31).

1.1.1. C. von Willebrand Factor type C domain

TSP1 and TSP2 contain a single VWC module, also known as chordin-like cysteine-rich repeat, or N-terminal fibrillar pro-collagen module. The atomic structure of this module has not been solved. However, insights can be gained from low resolution biophysical studies of the VWC module of TSP1 (32). The VWC domain contains 10 cysteine residues, which do not have high sequence conservation, with some exceptions at the second and eighth cysteines (33). Mass spectrometry analysis of the VWC of TSP1 revealed that this module is glycosylated and that all the cysteine residues are involved in disulfide bonds that stabilize the protein.

1.1.1. D. Thrombospondin repeats

TSP1 and TSP2 contain three tandem TSRs. TSRs (about 60 amino acids long) are conserved over evolution, and contain around 12 conserved residues comprising 6 cysteines, 2 conserved arginine residues, and 2 to 3 tryptophan residues separated by 2 to 4 amino acids (34, 35). Two unsual types of glycosylation have been recognized in

the TSRs. C-mannosylation of tryptophan residues is co-tranlational and is mediated by dolichol-P-mannose. It occurs within the TSR WXXW sequence (36). O-fucosylation is a post-translational modification mediated by O-fucosyltransferase-2 that occurs within the CSX(S/T)C sequence of TSRs (serine or threonine residues) (37), and it is critical for protein maturation and secretion (37, 38).

1.1.1. E. Signature domain

This portion of the protein includes the tandem EGF-like repeats, 13 Ca^{2+} binding repeats comprising the Ca^{2+}-binding wire, a C-terminal lectin-like module. The largest difference in the signature domains among TSPs is in the number of EGF-like repeats (3 in TSP1 and TSP2) (1). EGF1 contains a site that is modified by a glucose-xylose disaccharide in bovine TSP1. EGF2 binds a single Ca^{2+} at the interface between EGF1 and EGF2 and likely stabilizes the interactions between the modules. EGF3 has interaction with EGF2, the N- and C-terminal portions of the Ca^{2+}-binding wire, and the lectin-like module. The Ca^{2+}-binding wire forms a stable, Ca^{2+}-dependent structure that is independent of the rest of the signature domain and transmits allosteric changes induced by Ca^{2+} ions to other parts of TSPs (3, 39). The Ca^{2+}-binding wire comprises 13 aspartate-rich repeats that form individual globular folds connected in a disulfide-stabilized arrangement. On average, each repeat binds two Ca^{2+} ions. Ca^{2+} binding causes a conformational change (40), and keeps the signature domains of TSPs in a more compact conformation.

The lectin-like module is composed of two β-sheets (each comprising seven β-strands). Of the two β-sheets, one has a convex curve on the surface of the protein and the other has a more severe bend toward the interior of the protein. A short α-helix lies within the cleft formed by the second β-sheet. Homology-searching algorithms based on structure, placed this module in the L-type lectin domain family.

1.1.2.Major sites of expression

TSP1 is expressed in various adult and developing tissues with a complex pattern. However, the major changes occur during pathophysiological conditions. TSP1 is rapidly and transiently released at high concentrations in response to tissue injury (6, 41). TSP1 is expressed in healing wounds of skin, skeletal (42), muscle, nerve (43, 44), atherosclerotic lesions, and intimal hyperplasia (45, 46). TSP1 is expressed in the hemostatic plug with a unique spatial-temporal distribution (47).

TSP1 is elevated in some chronic coronary artery diseases (48). TSP1 is expressed by corneal and iris fibroblasts in pseudoexfoliation syndrome (49). TSP1 is abundantly expressed in the rheumatoid synovium (50). TSP1 is highly expressed by stromal fibroblasts and endothelial cells within tumors during the early stages of tumor progression (4), maybe as a host anti-tumor defense mechanism (5). However, TSP1 expression is frequently lost during tumor progression. This is due to positive regulation by tumor suppressors such as p53 (51) and negative regulation by activated oncogenes such as Ras, Myc, and Id1 (52-54).

1.1.3.Molecular Connections

TSP1 binds to other extracellular molecules such as matrix components, proteases, cytokines, and growth factors. TSP1 also binds to transmembrane glycoproteins that can serve as signalling receptors for TSP1. The binding sites for these receptors have been mapped using synthetic peptides. The N-terminal domain of TSP1 contains consensus high-affinity BBXB heparin/HSPG-binding motifs. The TSRs contribute to heparin binding with multiple lower-affinity sites (55, 56). TSP1 also binds decorin (57), activated α3β1, α6β1, α9β1, and α4β1 integrins (3, 58-60), fibrinogen (29), calreticulin (3), and LRP (61) through the N-terminal domain. The TSRs mediate interactions of TSP1 with CD36 (62, 63), β1 integrins (64, 65), fibronectin (66), matrix metalloproteinase 2 (MMP-2) (67), and TGF-β1 (68). The WSHWSPW motif in the second type 1 repeat of TSP1 binds to the latent TGF-β1. Mature active TGF-β1 is then released by an intermolecular activation effect of the KRFK motif in the first type 1 repeat of TSP1 (68). This interaction with TGF-β1 is relevant for the homeostatic function of TSP1. The wire of TSP1 contains binding motifs for αvβ3 and αIIbβ3 integrins (69), cathepsin G, and neutrophil elastase (70). The C-terminal lectin-like module of TSP1 contains the CD47-binding site (71).

Often, the binding of TSP1 modulates the activity of the binding partner. For example, binding of TSP1 to neutrophil cathepsin G, and elastase, or MMP-2 decreases the catalytic activity of these enzymes. This process may be important in collagen fibrillogenesis and the regulation of angiogenesis.

Differential expression or activation of cell surface receptors for TSP1, including integrins, CD36, CD47, LRP, proteoglycans, and sulfatides, may dictate the specific responses of each cell type to TSP1 (72-74).

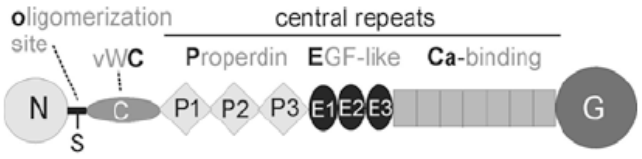
1.1.4.Roles of Thrombospondin-1

Gene knockout mice and human genetic diseases provide evidences of the context-specific roles of TSP1 *in vivo*. Mice null for TSP1 were described in 1998 (75). TSP1 gene knockout mice are fertile and capable of normal development. However, the embryonic viability is decreased, and the phenotypes include pneumonia (from 1 month of age), a mild spinal lordosis, and a two-fold increase in circulating monocytes.

The pneumonia, and abnormal inflammation in the lungs and pancreas of TSP1-null mice have many phenotypic similarities with those of TGF-β1-null mice (76), and are a consequence of a specific lack of TGF-β1 activation in these tissues. Although TSP1 is not the only activator of TGF-β1, it seems to have a dominant role in TGF-β1 activation in these two tissues, required for normal epithelial homeostasis and control of immune responses (77). Besides these minor defects, the null mice appear relatively normal. However, additional functions of TSP1 have been recently revealed by subjecting these mice to specific physiological stresses (78).

Functional studies revealed that TSP1 has many effects on cell function, including adhesion, cytoskeletal organization, migration, neurite outgrowth, cell-cell aggregation, proliferation, apoptosis, differentiation, and transcriptional regulation (**Figure 4**).

Figure 4. Schematic diagram of the molecular interactions and functions of Thrombospondin-1 subunits



Molecular interactions	Calreticulin α4β1, α3β1, α6β1, α9β1 integrins Fibrinogen HSPG/Heparin Decorin LRP	CD36 Fibronectin, TGF-β, MMP-2, β1 integrins HSPG	αvβ3, αIIbβ3 integrins Cathepsin G Elastase	CD47
Functions	Cell attachment, spreading, migration, platelet aggregation Endocytic uptake Focal adhesion disassembly Regulation of proliferation	Cell attachment Matrix interactions Neurite outgrowth Regulation of endothelial cells (inhibition of proliferation and angiogenesis, induction of apoptosis) Platelet aggregation (inhibition of NO-stimulated delay in aggregation) Regulation of epithelial homeostasis, immune response (pancreas, lungs)	Cell attachment, spreading Protease inhibition	Cell attachment, migration, platelet aggregation Smooth muscle cell proliferation T cell activation Inhibition of NO signalling

TSP1 supports the attachment of many cell types. After the attachment, the cells can either remain round and static or spread and become motile. Cell spreading and migration on TSP1 substrata are associated with an organization of the cortical cytoskeleton that involves the formation of large lamellipodia (79). The effects of TSP1 on the actin cytoskeleton are diverse and cell-type dependent. Glomerular mesangial cells, monocytes, neutrophils, smooth muscle, skeletal myoblasts, and carcinoma cells, are some examples of cells that spread and are motile on TSP1. The effects of TSP1 in vascular smooth muscle cells (VSMC) motility are relevant in the context of atherosclerotic lesions and vascular injury. VSMC chemotaxis to TSP1 depends on CD47, αv-type integrin (80), and β1 integrins (81). Treatment of injured arteries with antibody to the CD47-binding site of TSP1 reduces neointimal thickening (82). In addition, polymorphisms in TSP1, TSP2 and TSP4, increase the risk for premature coronary artery disease, presumably due to an increased proatherogenic or prothrombotic activity (83). However, examination of larger populations have recently questioned the significance of this association (84).

Moreover, TSP1 potently antagonizes nitric oxide (NO)-driven VSMC relaxation, suggesting a role for TSP1 in controlling tissue blood flow (85). After acute ischemic injury, TSP1- and CD47-null mice show increased tissue survival (86, 87). TSP1 also promotes cell-cell adhesion or aggregation. TSP1 participates in the attachment of malarial-parasitized red blood cells (RBCs) to vascular endothelium (88). TSP1 binds directly to sickle RBCs (89) and participates in the adhesion of sickle RBCs to endothelial cells *in vitro* (90), contributing to the pathogenesis of vaso-occlusion in sickle disease. In addition, TSP1 plays a role in platelet function (platelet aggregation and fibrin clot formation). One pathway through which TSP1 modulates platelet function is by preventing von Willebrand factor degradation by the plasma protease ADAMTS-13. This manifests as defective thrombus adherence in TSP1-null mice (91). A second pathway through which TSP1 modulates platelet function is by antagonizing the physiological anti-thrombotic activity of NO (92). NO plays an important role in limiting the aggregation of platelets (93). TSP1, and recombinant fragments from the type 1 repeats and the C-terminal domain, inhibit the NO-stimulated delay in aggregation by a mechanism that involves inhibition of multiple steps in the NO/cGMP signalling cascade (92).

The effects of TSP1 on cell proliferation are also cell-type specific. For example, TSP1 synergizes with growth factors such as EGF or PDGF to promote proliferation of SMC (94). For endothelial cells, TSP1 inhibits proliferation and actively induces apoptosis (95-97). CD36 is proposed to be a critical receptor because TSP1 does not show anti-angiogenic activity in the cornea in a CD36-null mouse model (98). However, the avascular cornea is a specialized tissue that is maintained by high expression of soluble vascular endothelial cell growth factor (VEGF) receptor-1 (99). This environment is permissive for fibroblast growth factor (FGF)-2 driven angiogenesis which is NO-independent, but not for NO-mediated VEGF angiogenesis. In contrast to the cornea, vascular outgrowth from CD36-null muscle explants remains sensitive to inhibition by TSP1 (87). Therefore, additional TSP1 receptors mediate this activity (100). Peptide motifs adjacent to the CD36-binding site also inhibit angiogenesis. These peptide motifs might enhance the binding of TSP1 to CD36, or could have indirect effects such as sequestration of pro-angiogenic factors (97, 101, 102). Although the interaction of TSP1 with CD36 plays a significant role in the anti-angiogenic activity of TSP1, CD36-independent mechanisms have also been identified. The TSRs can bind β1 integrins (64) and stimulate endothelial cell migration (103).

CD47 has been reported to correlate with increased endothelial cell apoptosis (104). CD47 binding peptides from the signature domain of TSP1 also inhibited angiogenesis in the mouse cornea (105). Vascular outgrowth from CD47-null muscle explants was not inhibited by TSP1, indicating that CD47 is necessary for an anti-angiogenic response to TSP1 in this assay (106). Furthermore, TSP1 and recombinant domains did not inhibit NO signalling in CD47 null endothelial cells. Finally, angiogenesis of full thickness skin grafts is enhanced in TSP1 null and CD47 null mice but not in CD36 null mice (107). Therefore CD47, not CD36, is the essential TSP1 receptor for inhibiting angiogenesis in ischemia.

In addition, TSP1 inhibits angiogenesis through effects on VEGF bioavailability. TSP1 suppresses the release of VEGF from the ECM by inhibiting the activation of MMP-9 (108). TSP1 also binds directly to VEGF and mediates its uptake and clearance (109). TSP1 activates latent TGF-β in the tumor microenvironment (110), and this activity may also contribute to tumor dormancy. TSP1 is often down-regulated during tumor progression (111) and this loss is linked to alterations in specific oncogenes and tumor suppressor genes (p53, Myc, Ras) that control TSP1 expression (51, 53).

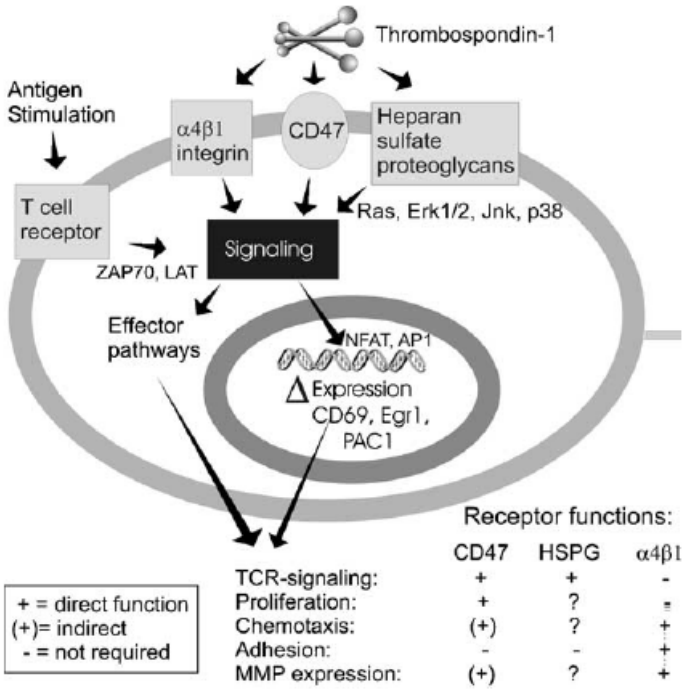
1.1.4.A. Regulation of Immune Responses

TSP1-null mice display a phenotype that includes acute and chronic inflammatory pulmonary infiltrates, and an elevated number of circulating white blood cells (77), suggesting an anti-inflammatory role. As previously described in page 53, the pneumonia and abnormal inflammation in the lungs and pancreas of TSP1-null mice may be a consequence of a specific lack of latent TGF-β1 activation in these tissues, required for normal epithelial homeostasis and control of immune response (77). One main function of TGF- β is to maintain T cell tolerance to self via its direct inhibition of T helper 1 (Th1), Th2, and cytotoxic T lymphocyte (CTL) differentiation and the maintenance of regulatory T (Treg) cells (112). Human natural killer cell proliferation is also regulated by TSP1-mediated activation of latent TGF-β1 (113). TGF-β1 is initially masked by its non-covalent association with a dimer of its N-terminal propeptide, called latency-associated protein (LAP) (114, 115). TSP1 seems to have a dominant role in latent TGF-β1 activation, especially in the lungs and pancreas.

The central type I repeats of TSP1 (116) interact with the N-terminal region of LAP, forming a trimolecular complex. Within the complex, a conformational change takes place and makes TGF-β1 accessible to its receptor (77).

In addition, TSP1 directly regulates T cell function. Studies using cDNA microarrays indicate that intact TSP1 is predominantly an inhibitor of T cell antigen receptor (TCR) signal transduction (117), although some peptides show different activities from that of intact TSP1. Type I repeat domains from TSP1 bind to HSPG on T cells and stimulate CD3-induced Ras activation and tyrosine phosphorylation of the three MAP kinases Erk1/2, JNK, and p38 (118). Somatic mutant T cell lines deficient in either β1 integrins or in CD47 were used to dissect the functions of these TSP1 receptors (119). α4β1 integrin is necessary and sufficient for stimulation of T cell adhesion, and it is also required for stimulation of chemotaxis and MMPs gene expression by TSP1 and TSP2 (119). CD47 plays a significant role in inhibiting TCR signalling and T cell proliferation (117), and inhibiting differentiation of naïve T cells into Th1 effectors (120) (**Figure 5**) (121).

Figure 5. Thombospondin-1 signal transduction in T lymphocytes
(Kuznetsova, S.A., et al., 2004)



CD47 null mice have defects in leukocyte motility and impaired responses to bacterial pathogens (122). This phenotype is consistent with evidence that CD47 controls activation of α4β1 (123), required for leukocyte diapedesis in response to some inflammatory signals. TSP1 may also regulate T cell function indirectly through its effects on antigen presenting cells (124, 125).

1.1.4.B. Regulation of Innate Immune Responses

ECM proteins released at the site of inflammation or injury may modulate polymorphonuclear leukocytes (PMNs) phagocytosis, adhesion, motility, oxidant generation, etc. TSP1 is predominantly stored in platelets but also secreted at lower levels by many cell types including monocytes, macrophages, and dendritic cells (DCs). TSP1 plays several roles in the physiological functions of phagocytes.

Apoptotic monocytes-derived TSP1 and its HBD, lead to immature DC tolerogenic and phagocytic state (124). TSP1 mediates phagocytosis of senescent eosinophils and neutrophils undergoing apoptosis by human monocyte-derived macrophages, as an injury-limiting disposal mechanism that involves αvβ3/CD36/TSP1 (126, 127). Macrophages mediate phagocytosis of apoptotic eosinophils by a mechanism uncoupled from chemokine secretion. By contrast, phagocytosis of post-apoptotic eosinophils may elicit pro-inflammatory responses (126).

Macrophage phagocytosis of apoptotic neutrophils can be potentiated by proinflammatory cytokines such as TGF-β1, and granulocyte-macrophage-colony-stimulating factor (GM-CSF), as a negative feedback control of neutrophil number at inflamed sites (127). Macrophage recognition and phagocytosis of apoptotic fibroblasts is dependent on fibroblast-derived TSP1 and CD36 (128). In addition, semiprofessional phagocytes such as mesangial cells mediate phagocytosis of apoptotic neutrophils by a mechanism dependent of αvβ3/TSP1, independent of CD36, and uncoupled from chemokine secretion (129).

TSP1 may regulate leukocyte function associated antigen (LFA)-1/intracellular cell adhesion molecule-1 (ICAM-1)-mediated homotypic aggregation of human monocytic U937 cells by either the inhibitory effect via CD47 or the promoting effect through CD36 (130). Moreover, TSP1 plays a crucial role in inflammation and atherogenesis by increasing monocyte attachment to endothelium. The mechanism involves up-regulation of vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 (131). Soluble TSP1 at high concentrations stimulates motility of human neutrophils (132), (133) and promotes chemotaxis and haptotaxis of human peripheral blood monocytes (134).

TSP1 may play a role in leukocyte differentiation and cytokine expression. TSP1 is involved in retinoic acid-induced differentiation of HL-60 myeloid leukemia cells (135). Ligation of CD47 by the TSP1-derived peptide 4N1K during monocyte differentiation into DC, reduces interleukin (IL)-12 and tumor necrosis factor-α (TNF-α) expression (136). Thus, DC-derived TSP1 serves as a negative regulator contributing to arrest of cytokine production, resolution of inflammation, and maintenance of steady state (125, 137). CD36-deficient patients have impaired response of oxidized low density lipoprotein (LDL)-induced NF-κB activation and subsequent cytokine expression (138). TSP1 modulates expression of IL-6 and IL-10 by monocytes (139) and activation of latent TGF-β (77). In addition, TSP1 binds to human neutrophils through the residues F16-G33 and A784-N823 (140), enhances cytokine-induced respiratory burst of human neutrophils (141) and enhances chemoattractant n-formyl-methionyl-leucyl-phenylalanine (FMLP)-mediated superoxide anion (O_2^-) generation from human neutrophils through its N-terminal domain (140, 142). However, the underlying mechanism for regulation of O_2^- generation has not been delineated.

Here, we provide evidence that soluble TSP1 causes a significant increase in phorbol 12-myristate 13-acetate (PMA)-mediated O_2^- generation from interferon- γ (INF-γ)-differentiated human monocytes by interaction with its receptor α6β1 integrin through its N-terminal region and identify a requirement for and extracellular calcium (Ca^{2+}) to mediate the macrophage respiratory burst.

1.1.4.C. Regulation of Macrophage-mediated Innate Immune Response against tumors

Macrophages are an important effector cell of innate immunity against tumors. However, tumor-associated macrophages (TAMs) can differentiate into either cytotoxic (M1) or tumor growth-promoting (M2) states. This differentiation depends on the tissue microenvironment (143). Macrophages are classically activated toward the M1 phenotype by IFN-γ alone or in concert with microbial products. Alternative activation by stimulation with IL-4 or IL-13, IL-10, IL-21, TGF-β, immune complexes, and glucocorticoids drives macrophages toward M2 phenotype (144).

In general, M2 macrophages are present in established tumors and promote tumor progression (145). TSP1 is often down-regulated during tumor progression and inhibits tumor growth when re-expressed. This activity is generally attributed to inhibiting angiogenesis, but the above results suggest that effects on tumor immunity should also be considered. The current study demonstrates an important role for TSP1 as a positive modulator of innate anti-tumor immunity by increasing macrophage recruitment and stimulating reactive oxygen species (ROS)-mediated tumor cytotoxicity.

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II. OBJETIVOS

II

A.OBJETIVOS

El propósito de este proyecto es determinar el mecanismo por el cual la proteína de matriz extracelular TSP1 regula la respuesta inmune innata. Numerosos estudios sugieren que la TSP1 juega un papel importante en el reclutamiento de monocitos y de macrófagos en tejidos que han sufrido algún tipo de daño y en focos inflamatorios. Además, en trabajos anteriores se ha observado un aumento en el infiltrado de macrófagos en tumores que sobreexpresan TSP1. Así pues, el objetivo es investigar el papel de la TSP1 en el reclutamiento de macrófagos en el tumor.

Nuestra hipótesis de partida es la siguiente: “La TSP1 podría estar jugando un papel relevante en la inmunidad anti-tumoral al aumentar el reclutamiento y la activación de los TAMs de fenotipo M1“; y se pretende validar respondiendo las siguientes preguntas:

¿Podemos estimular el reclutamiento de los TAMs de fenotipo M1 *in vivo* mediante la sobreexpresión de TSP1?.

¿Es la TSP1 capaz de potenciar la activación de los macrófagos de fenotipo M1 *in vitro* (citotoxicidad, producción de ROS y de citoquinas y quimioquinas)?.

B.STATEMENT OF PURPOSE

The purpose of this study was to determine the regulation of innate immune responses by the ECM protein TSP1. Since several studies have suggested that TSP1 plays an important role in the recruitment of monocytes and macrophages to sites of tissue injury or inflammation, and previous reports have shown that tumors over-expressing TSP1 are heavily infiltrated in macrophages, the role of TSP1 in macrophage recruitment into the tumor was investigated.

The following hypothesis was tested: “TSP1 may play an important role in anti-tumor immunity by enhancing recruitment and activation of M1 TAMs”, by asking the following relevant questions.

Does TSP1 over-expression stimulate M1 TAMs recruitment *in vivo*?

Can TSP1 enhance activation of M1 macrophages (tumor cytotoxicity, ROS generation, production of cytokines and chemokines) *in vitro*?

III. RESULTS

III

A.RESULTS

Gema Martin-Manso, Susana Galli, Lisa A. Ridnour, Maria Tsokos, David A. Wink, and David D. Roberts. Thrombospondin1 promotes Tumor Macrophage Recruitment and Enhances Tumor Cell Cytotoxicity of Differentiated U937 Cells. *Cancer Res*. 2008; 68(17): 7090-9

Thrombospondin 1 Promotes Tumor Macrophage Recruitment and Enhances Tumor Cell Cytotoxicity of Differentiated U937 Cells

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Abstract

Inhibition of tumor growth by thrombospondin (TSP) 1 is generally attributed to its antiangiogenic activity, but effects on tumor immunity should also be considered. We show that overexpression of TSP1 in melanoma cells increases macrophage recruitment into xenograft tumors grown in nude or beige/nude mice. *In vitro*, TSP1 acutely induces expression of plasminogen activator inhibitor-1 (PAI-1) by monocytic cells, suggesting that TSP1-induced macrophage recruitment is at least partially mediated by PAI-1. Tumor-associated macrophages (TAM) can either promote or limit tumor progression. The percentage of M1-polarized macrophages expressing inducible nitric oxide synthase is increased in TSP1-expressing tumors. Furthermore, soluble TSP1 stimulates killing of breast carcinoma and melanoma cells by IFN-γ–differentiated U937 cells *in vitro* via release of reactive oxygen species. TSP1 causes a significant increase in phorbol ester–mediated superoxide generation from differentiated monocytes by interaction with $\alpha_6\beta_1$ integrin through its NH_2-terminal region. The NH_2-terminal domain of TSP2 also stimulates monocyte superoxide production. Extracellular calcium is required for the TSP1-induced macrophage respiratory burst. Thus, TSP1 may play an important role in antitumor immunity by enhancing recruitment and activation of M1 TAMs, which provides an additional selective pressure for loss of TSP1 and TSP2 expression during tumor progression. [Cancer Res 2008;68(17):7090–9]

Introduction

Thrombospondin 1 (TSP1) is a secreted glycoprotein that is predominantly stored in platelets but also secreted at low levels by many cell types, including monocytes and macrophages. TSP1 is rapidly and transiently released in response to tissue injury and is elevated in several chronic diseases (1, 2). TSP1-null mice display acute and chronic inflammatory pulmonary infiltrates and an elevated number of circulating WBCs (3), suggesting an anti-inflammatory role. In contrast, TSP1 expression in ischemic injuries limits tissue survival and restoration of perfusion by blocking nitric oxide/cyclic guanosine 3′,5′-monophosphate signaling (4). The diverse biological activities of TSP1 are mediated by its multiple functional domains that engage corresponding receptors expressed by a variety of cells. Differential expression or activation of cell surface receptors for TSP1, including integrins, CD36, CD47, low-density lipoprotein (LDL) receptor-related protein, proteoglycans, and sulfatides, may dictate the specific responses of each cell type to TSP1 (5).

TSP1 plays several roles in the physiologic functions of phagocytes. TSP1 mediates phagocytosis of neutrophils undergoing apoptosis (6). Macrophage recognition and phagocytosis of apoptotic fibroblasts requires fibroblast-derived TSP1 and CD36 (7). CD36-deficient patients have impaired oxidized LDL-induced nuclear factor-κB activation and subsequent cytokine expression (8). TSP1 modulates expression of interleukin (IL)-6 and IL-10 by monocytes (9) and activation of latent transforming growth factor β (TGFβ; ref. 3). TSP1 stimulates motility of human neutrophils (10, 11) and promotes chemotaxis and haptotaxis of human peripheral blood monocytes (12). In addition, TSP1 enhances cytokine-induced respiratory burst of human neutrophils (13) and enhances chemoattractant fMLP-mediated superoxide anion (O_2^-) generation by human neutrophils through its NH_2-terminal domain (14, 15). However, the underlying mechanism for regulation of O_2^- generation has not been delineated. Here, we provide evidence that soluble TSP1 causes a significant increase in phorbol 12-myristate 13-acetate (PMA)–mediated O_2^- generation from IFN-γ–differentiated human monocytes by interaction with $\alpha_6\beta_1$ integrin through its NH_2-terminal region and identify a requirement for extracellular calcium to mediate the macrophage respiratory burst.

Macrophages are an important effector cell of innate immunity against tumors. However, tumor-associated macrophages (TAM) can differentiate into either cytotoxic (M1) or tumor growth–promoting (M2) states. This differentiation depends on the tissue microenvironment (16). Macrophages are classically activated toward the M1 phenotype by IFN-γ alone or in concert with microbial products. Alternative activation by stimulation with IL-4 or IL-13, IL-10, IL-21, TGFβ, immune complexes, and glucocorticoids drives macrophages toward the M2 phenotype (17). M2 macrophages are present in most established tumors and promote tumor progression (18).

TSP1 is often down-regulated during tumor progression and inhibits tumor growth when reexpressed (19). This activity is generally attributed to angiogenesis inhibition, but the above results suggest that effects on tumor immunity should also be considered. The current study shows an important role for TSP1 as a positive modulator of innate antitumor immunity by increasing M1 macrophage recruitment and stimulating reactive oxygen species (ROS)-mediated tumor cytotoxicity.

Materials and Methods

Proteins and peptides. Human TSP1 was purified from the supernatant of thrombin-activated platelets obtained from the NIH Blood Bank (20). Recombinant human TSP1 was obtained from EMP

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Genetech. Recombinant proteins containing various domains of TSP1 and TSP2 were prepared as previously described and provided by Dr. Deane Mosher (University of Wisconsin, Madison, WI; refs. 21–23). The $\alpha_6\beta_1$ integrin inhibitory peptide (LALERKDHSG) derived from TSP1 and the control peptide (LALARKDHSG) were prepared as described (24). Xanthine, xanthine oxidase, and superoxide dismutase (SOD) were obtained from Stratagene.

Reagents. Rat anti-mouse CD68 antibody (clone FA-11) was from AbD Serotec. Monoclonal neutralizing antibody (clone 9016) against human TGFβ1, recombinant human and mouse IFN-γ, recombinant human TGFβ1, and recombinant human IL-4 were from R&D Systems. Rabbit polyclonal to plasminogen activator inhibitor-1 (PAI-1) and rabbit polyclonal to inducible nitric oxide synthase (iNOS) were from Abcam, Inc. Anti-actin (Ab-1) mouse monoclonal antibody and EGTA were from Calbiochem. The function-blocking rat anti-human α_6 integrin monoclonal antibody (clone G0H3) was from Chemicon International, Inc. FITC-conjugated rat anti-human α_6 monoclonal antibody (clone G0H3) and the isotype control were from BD Biosciences. Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 and PMA were from Sigma-Aldrich. The inhibitor of iNOS, aminoguanidine, was from Sigma-Aldrich. The $\alpha_4\beta_1$ integrin antagonist [4-((2-methylphenyl)aminocarbonyl)aminophenyl] acetyl-LDVP (25) was obtained from Bachem. The calcium indicator Fluo-4/AM, rabbit polyclonal anti-fluorescein/Oregon Green, and Pluronic F-127 were from Molecular Probes.

***THBS1*-transfected cells.** MDA-MB-435 cells transfected with the *THBS1* expression plasmid (clone TH26, 7.5-fold higher TSP1 expression than control) or the empty pCMVBamNeo vector (Neo) were described previously (26).

Cell culture and differentiation. Transfected MDA-MB-435 cells were cultured at 37°C, 5% CO_2, in complete RPMI 1640 (Life Technologies) containing 10% fetal bovine serum (FBS; Biosource), 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 750 μg/mL geneticin (Life Technologies). The human monocytic line U937 (27), kindly provided by Dr. Mark Raffeld [National Cancer Institute (NCI), NIH, Bethesda, MD], was cultured at 37°C, 5% CO_2, in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% endotoxin-tested FBS (Biosource). For differentiation with IFN-γ, 2.0×10^5/mL U937 cells in complete growth medium containing 1 mmol/L sodium pyruvate, 0.1 mmol/L MEM with nonessential amino acids (Cellgro), and 100 units/mL recombinant human IFN-γ were incubated for 3 d at 37°C. For differentiation with IL-4, 2.0×10^5/mL U937 cells in AIM-V + Albumax serum-free medium (Life Technologies) containing 10 ng/mL recombinant human IL-4 were incubated for 3 d at 37°C. MDA-MB-231, MDA-MB-435, and MCF-7 cells were cultured in RPMI 1640 containing 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Murine macrophage cell lines ANA-1 (28) and RAW264.7 were cultured in DMEM (Life Technologies) supplemented with 2 mmol/L glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% endotoxin-tested FBS. Human peripheral blood mononuclear cells (PBMC) were prepared by gradient centrifugation. In brief, fresh human buffy coat (NIH Blood Bank) was diluted 1:4 with sterile Dulbecco's PBS (Life Technologies). Human PBMCs were isolated by mixing 1.077 g/mL Lymphocyte Separation Medium (Cambrex) and the diluted human blood and centrifuged for 30 min at $900 \times g$, 18°C to 20°C. Human monocytes were isolated from PBMCs by adherence to plastic.

Tumorigenesis assay in nude mice. Groups of 10 female NIH-*bg/nu* mice, ~8 wk of age, were injected in the mammary fat pads with 8×10^5 Neo or TH26 cells. Primary tumor size was determined twice weekly by length × width × height measurement. The primary tumors were removed on week 11.

A total of 15 female NIH-*nu/nu* mice, 7 wk of age, were s.c. injected in the right hind leg with 5×10^6 MDA-MB-435 cells. Five animals were injected with Neo cells and 10 with TH26 cells. Primary tumor size was determined twice weekly, and tumor volume was calculated as $(\text{width})^2 \times \text{length}/2$. Primary tumors were removed when the volume was 300 to 400 mm^3 or at week 7. For histopathologic analysis, tumor tissues were fixed in buffered formalin, embedded in paraffin, sectioned (5 μm), and stained with H&E. Animal experiments were conducted in an accredited facility according to NIH guidelines under a protocol approved by the NCI Animal Care and Use Committee.

Immunohistochemical evaluation. Slides were deparaffinized in xylene (thrice for 10 min) and rehydrated in graded alcohol (100%, 95%, and 70%). Antigen retrieval was performed in a pressure cooker containing Target Retrieval Solution (pH 6.10; Dako Corp.) for 30 min (CD68 antibody) or 10 min (PAI-1 antibody) or 10 mmol/L citrate buffer (pH 6.0) for 10 min, followed by cooling at room temperature for 20 min (iNOS antibody), and then washed with PBS twice for 10 min. Endogenous peroxidase activity was quenched by 0.3% H_2O_2 in water. After washing the slides with Wash Buffer Solution (Dako), nonspecific binding was reduced using Protein Block Serum-Free (Dako) for 10 min. The slides were incubated with CD68 antibody (1:100, overnight at 4°C), PAI-1 antibody (1:250 dilution, 1 h at room temperature), and iNOS antibody (1:50, 1 h at room temperature). Slides were then incubated with streptavidin-biotin (Dako LSBA+ kit, horseradish peroxidase). 3,3′-Diaminobenzidine (Dako) was used as chromogen for 5 min, and hematoxylin was used for counterstaining. Negative control slides omitted the primary antibody. CD68 was located predominantly within the cells. Nuclei were negative. Cytoplasmic and extracellular staining in macrophages was considered positive for PAI-1. Cytoplasmic staining in macrophages was considered positive for iNOS. The intensity of the staining was evaluated using a Nikon Eclipse E1000 microscope equipped with a microcolor camera (RGB-MS-C). The acquisition software was IPLab-Scientific Image Processing 3.5.5.

Measurement of monocyte chemotactic protein-1, PAI-1, and IL-10. Monocyte chemotactic protein-1 (MCP-1), PAI-1, and IL-10 levels in differentiated U937 cell supernatants were measured with a multiplex ELISA array (Quansys Biosciences). All samples were run in replicate.

Western blotting. RAW264.7 cells were serum deprived for 48 h before addition of TGFβ1 or TSP1. After 2 to 4 h of incubation at 37°C, 5% CO_2, in AIM-V + Albumax serum-free medium, cells were lysed at 4°C in 0.5% deoxycholic acid, 0.1% SDS, 50 mmol/L HEPES, 1% Triton X-100, 1% NP40, 150 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Cell pellets were vortexed briefly and centrifuged at 14,000 rpm for 15 min. Cell lysates (15 μg protein) were boiled for 5 min in SDS sample buffer, electrophoretically separated on NuPAGE 10% Bis-Tris gels (Invitrogen) for 1.5 h at 150 V, and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) for 2 h at 100 V. Membranes were blocked in 5% bovine serum albumin (BSA)/0.1% Tween 20/PBS and incubated overnight with rabbit polyclonal to PAI-1 (2.5 μg/mL). Enhanced chemiluminescence (Upstate) was used for detection. Stripped membranes were reprobed with actin antibody to confirm protein loading levels.

Real-time quantitative reverse transcription-PCR analysis. Total RNA was extracted from Neo and TH26 tumors using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was treated with recombinant DNase I (DNA-free kit, Applied Biosystems) and quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). cDNA was synthesized from total RNA using iScript cDNA Synthesis kit (BioRad). Real-time PCR for mouse iNOS and arginase-1 expression profiling was performed on a 7500 Real-Time PCR instrument (Applied Biosystems) using Taqman oligonucleotide primers Mm00440485_m1 and Mm00475988_m1, respectively. Data were normalized against mouse hypoxanthine phosphoribosyltransferase 1 (HPRT1; Mm00446968_1). Relative iNOS and arginase-1 expression was calculated using the $2^{-\Delta\Delta CT}$ method.

U937 and ANA-1 cell-mediated cytolysis. MDA-MB-231, MDA-MB-435, and MCF-7 target cells were seeded into 16-well plates in 150 μL of growth medium. Cell growth was dynamically monitored using RT-CES system (ACEA Biosciences) for 24 h. Differentiated U937 effector cells at an E:T ratio of 40:1 were added into wells containing target cells. ANA-1 cells were activated for 20 h at 37°C with 10 ng/mL of LPS and 100 units/mL of IFN-γ in complete medium and also used at an E:T ratio of 40:1. After addition of effector cells, measurements were automatically collected by the analyzer every 10 min for up to 48 h.

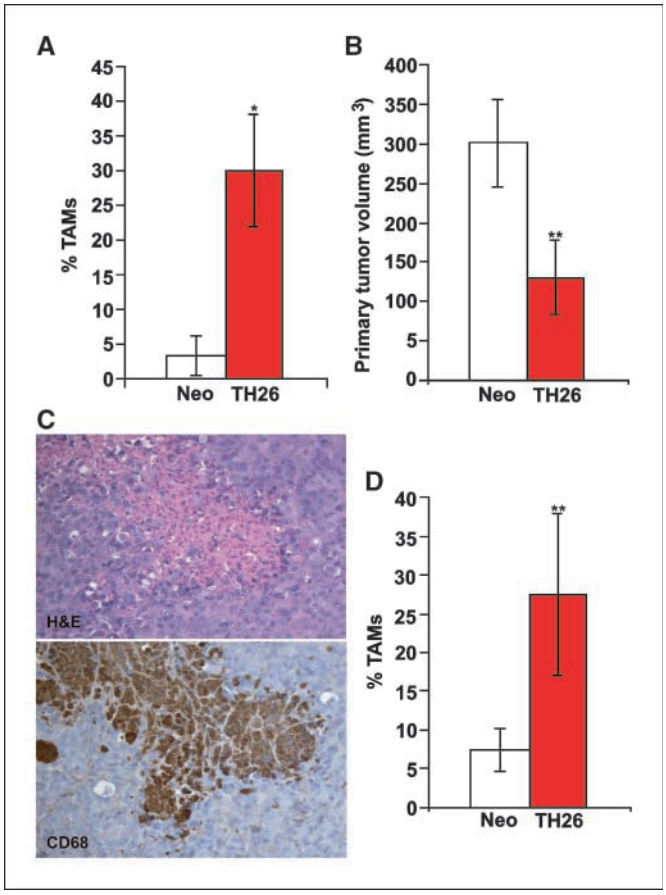

Figure 1. *THBS1* overexpression promotes macrophage recruitment in tumor-bearing nude mice. *A,* tissue sections cut from paraffin-embedded tumors harvested from NIH-*bg/nu* mice injected in the mammary fat pads with TH26 or Neo MDA-MB-435 clones were stained with H&E and rat anti-mouse CD68 antibody (clone FA-11). Quantitative analysis of macrophage infiltration into the tumor specimens was performed by evaluating the number of CD68-positive cells per 100× field in nonnecrotic areas. The results are presented as a percentage of TAMs in Neo control (*white column*) versus *THBS1*-transfected TH26 tumors (*red column*). *B,* primary tumor size 24 d after injection in NIH-*nu/nu* mice given s.c. injections of TH26 (*red column*) or Neo cells (*white column*). Histogram represents the tumor volume (mm^3) of all animals within each group that developed a tumor. *C,* paraffin-embedded sections cut from clone TH26 tumors grown s.c. in NIH-*nu/nu* mice were stained with H&E (*top*) and rat anti-mouse CD68 antibody (clone FA-11; *bottom*). Representative photomicrographs of adjacent slides are shown from experiments conducted in tumor samples from 12 NIH-*nu/nu* mice. Three mice did not develop tumors. Identical patterns were observed in all of the tumors examined. Magnification, ×200. *D,* quantitative analysis of macrophage infiltration into s.c. tumors grown in NIH-*nu/nu* mice was evaluated as the percentage of CD68-positive cells in multiple 100× fields in nonnecrotic areas. The results are presented as a percentage of TAMs in Neo (*white column*) and TH26 tumors (*red column*).

Cytotoxicity assay. MDA-MB-231 cells (2×10^4 per well) were seeded into 96-well plates in 200 μL of growth medium in the presence or absence of soluble TSP1 for up to 72 h at 37°C. MDA-MB-231 cells (2,500 per well) were seeded into 96-well plates in 100 μL of RPMI 1640 containing 1.25% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in the presence or absence of xanthine/xanthine oxidase for 72 h at 37°C. Media were collected to assess lactate dehydrogenase (LDH) released due to cell death. LDH release was quantified using a colorimetric assay (Promega). All samples were run in triplicate.

Superoxide production. O_2^- levels in differentiated U937 and activated ANA-1 cell supernatants were quantified using the LumiMax Superoxide Anion Detection kit (Stratagene).

Flow cytometry analysis. Direct immunofluorescence was performed by incubating 1×10^6 cells with 50 μg/mL of FITC-conjugated rat anti-human α_6 antibody (clone G0H3) or isotype control for 45 min at 4°C in HBSS containing 0.1% BSA and 0.1% sodium azide (Sigma-Aldrich). After staining, propidium iodide was added and the cells were analyzed on a FACScan flow cytometer (Becton Dickinson). The analysis software was FlowJo (7.2.1).

Measurement of intracellular free Ca^{2+}. Differentiated U937 cells were incubated with loading solution consisting of HEPES-buffered saline [11.6 mmol/L HEPES (Cellgro) in HBSS] supplemented with 2 μmol/L Fluo-4, 0.02% Pluronic F-127, and 1% BSA for 30 min and then incubated in loading solution without Fluo-4 for 30 min to allow deesterification of the probe. Loading solution was replaced with HBSS containing anti-fluorescein antibody and TSP1. The cells were then placed in a fluorometer (GENios Plus Tecan), and measurements were collected every 5 min for up to 40 min.

Statistical analysis. All data are shown as mean ± SD except were indicated. Significance was determined with one-tailed distribution Student's *t* test analysis. The difference was considered significant when $P \leq 0.05$ (*) and $P \leq 0.001$ (**).

Results

TSP1 overexpression increases tumor macrophage recruitment *in vivo*. Expression of TSP1 in the melanoma cell line MDA-MB-435 significantly inhibits tumor growth and metastasis (26). Notably, increased infiltration of monocytes was observed in clone TH26 tumors that highly express TSP1 (26) and in tumor-bearing mice treated with TSP1 peptides (29). Because macrophage recruitment into wounds is TSP1 dependent (1), we examined the effect of tumor cell TSP1 over expression on TAM recruitment. Using the previously described conditions of injection in the mammary fat pads (26), we confirmed that TH26 tumors showed delayed growth in female NIH-*bg/nu* mice relative to control-transfected clones (data not shown). The primary tumors were removed on week 11, and seven randomly selected mice were analyzed by immunohistochemistry. TAMs were detectable in nonnecrotic areas of tumor stained with H&E (data not shown). Based on CD68 antibody staining, the percentage of TAMs was significantly higher in TH26 tumors than in Neo tumors ($P < 0.05$; Fig. 1*A*).

Mammary fat pad injection was used previously as an orthotopic model of breast carcinoma growth, but recent studies have confirmed that the MDA-MB-435 cell line is a derivative of the M14 melanoma cell line (30). To determine whether TSP1 suppresses tumor growth at a site more appropriate for melanoma, 15 female NIH-*nu/nu* mice were s.c. injected in the right hind limb with TH26 or Neo cells. TSP1 overexpression in TH26 cells also inhibited s.c. tumor growth ($P \leq 0.001$; Fig. 1*B*). The primary tumors were removed when the volume was 300 to 400 mm^3 or at week 7 and analyzed by immunohistochemistry. Sections of tumor were stained with H&E and anti-mouse CD68 antibody. Increased TAM infiltration was observed in s.c. TH26 versus Neo tumors ($P < 0.001$; Fig. 1*C* and *D*).

Role of MCP-1 and PAI-1 in TSP1-dependent TAM recruitment. MCP-1 is an important regulator of monocyte recruitment (31), and a deficit in MCP-1 was proposed to account for decreased infiltration of macrophages into an excisional wound of TSP1-null mice (1). However, 12 h of stimulation with different doses of soluble TSP1 resulted in no significant change in total MCP-1 release from differentiated U937 human monocytic cells (Fig. 2*A, left*).

Macrophage migration *in vitro* and *in vivo* also depends on PAI-1 (32, 33), and elevated PAI-1 expression was previously

reported in TH26 versus Neo cells (34). Therefore, increased PAI-1 could also account for increased TAM recruitment into TH26 tumors. Because this result was obtained using a stable transfectant, it was unclear whether TSP1 directly regulates PAI-1 expression rather than the increased PAI-1 being an indirect adaptation of the transfected clones. Incubation of differentiated U937 cells with TSP1 resulted in an acute dose- and time-dependent increase in PAI-1 expression, with a maximal 50-fold

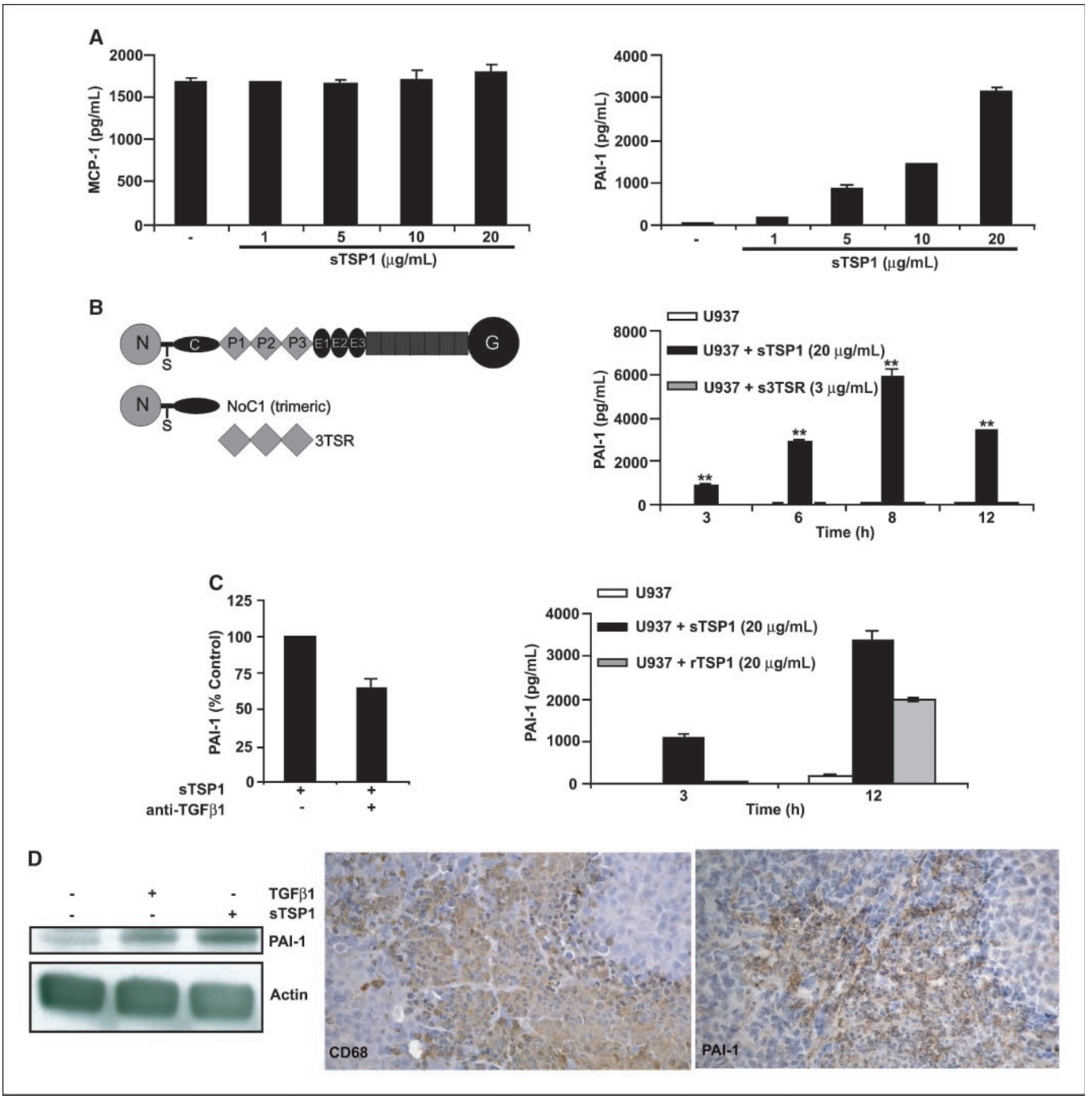

Figure 2. Effects of TSP1 on MCP-1 and PAI-1 expression in differentiated U937 human monocytic cells and mouse macrophages. *A* to *C,* differentiated U937 cells (1×10^6/0.5 mL RPMI 1640 with 0.5% FBS) were incubated in the presence or absence of soluble TSP1, soluble recombinant type 1 repeats of TSP1 (*3TSR*; *B*), or soluble recombinant human TSP1 (*C, right*). After 12 h of incubation (*A*), or at the indicated times (*B* and *C, right*), the supernatants were harvested, and total MCP-1 and PAI-1 were determined using a multiplex ELISA array as described in Materials and Methods. Data are representative of at least four different experiments. *C, left,* differentiated U937 cells were incubated with neutralizing TGFβ1 antibody (clone 9016) for 15 min before the addition of soluble TSP1 (20 μg/mL). Culture supernatants collected after 12 h were used to measure total PAI-1. Data are representative of two different experiments. *D, left,* serum-deprived murine RAW264.7 cells were stimulated with TGFβ1 (1 ng/mL), as a positive control, or soluble TSP1 (20 μg/mL) for 2 h. The experiment was repeated thrice, and a representative anti–PAI-1 blot is shown. Actin was used to confirm equal protein loading. *Right,* paraffin-embedded sections cut from TH26 tumors grown s.c. in NIH-*nu/nu* mice were stained with rat anti-mouse CD68 antibody (clone FA-11; *left*) and rabbit anti-mouse PAI-1 antibody (*right*). Representative photomicrographs of adjacent sections are shown from experiments conducted in tumor samples from 12 NIH-*nu/nu* mice. Identical patterns were observed in all of the tumors examined. Magnification, ×200.

induction at 8 h [$P < 0.001$; Fig. 2*A* (*right*) and *B*]. Because *PAI-1* is a TGFβ-inducible gene (35) and bioactive TGFβ is present in TSP1 purified from platelets (36), we asked whether TGFβ contamination or TSP1-mediated activation of latent TGFβ (3) contributed to the PAI-1 response in U937 cells. Interestingly, stimulation with 3 μg/mL of soluble recombinant 3TSR, the type 1 repeat domain of TSP1 responsible for the TSP1-mediated activation of latent TGFβ (37), resulted in no significant change in total PAI-1 release from differentiated U937 cells (Fig. 2*B*). Therefore, activation of latent TGFβ probably does not account for the effect of TSP1 on PAI-1 expression. The NH_2-terminal module of TSP1 increases fMLP-mediated O_2^- generation and chemotaxis by human neutrophils (10, 14) but also had no effect in PAI-1 expression (data not shown).

TSP1 stimulation of PAI-1 production was then examined in the presence of a neutralizing TGFβ1 antibody. At 5 μg/mL, the neutralizing antibody partially inhibited TSP1-stimulated PAI-1 production (Fig. 2*C, left*). Furthermore, recombinant human TSP1 at the same concentration, which should lack TGFβ contamination, showed less stimulatory activity than platelet TSP1 (Fig. 2*C, right*). Therefore, bioactive TGFβ present in platelet TSP1 at least partially mediates the stimulation of PAI-1 production by TSP1, but TSP1 lacking TGFβ is also active.

To address whether TSP1 also induces PAI-1 production in mouse macrophages, we used the RAW264.7 macrophage cell line. Increased PAI-1 expression was detected in whole-cell lysates within 2 h after TGFβ (positive control) or TSP1 treatment (Fig. 2*D, left*). Furthermore, increased PAI-1 secretion was detected in cell culture supernatants within 4 h after TGFβ or TSP1 treatment (data not shown). We also examined whether PAI-1 is expressed by murine TAMs *in vivo*. Immunohistochemical analysis showed strong PAI-1 staining in the TH26 TAMs (Fig. 2*D, right*).

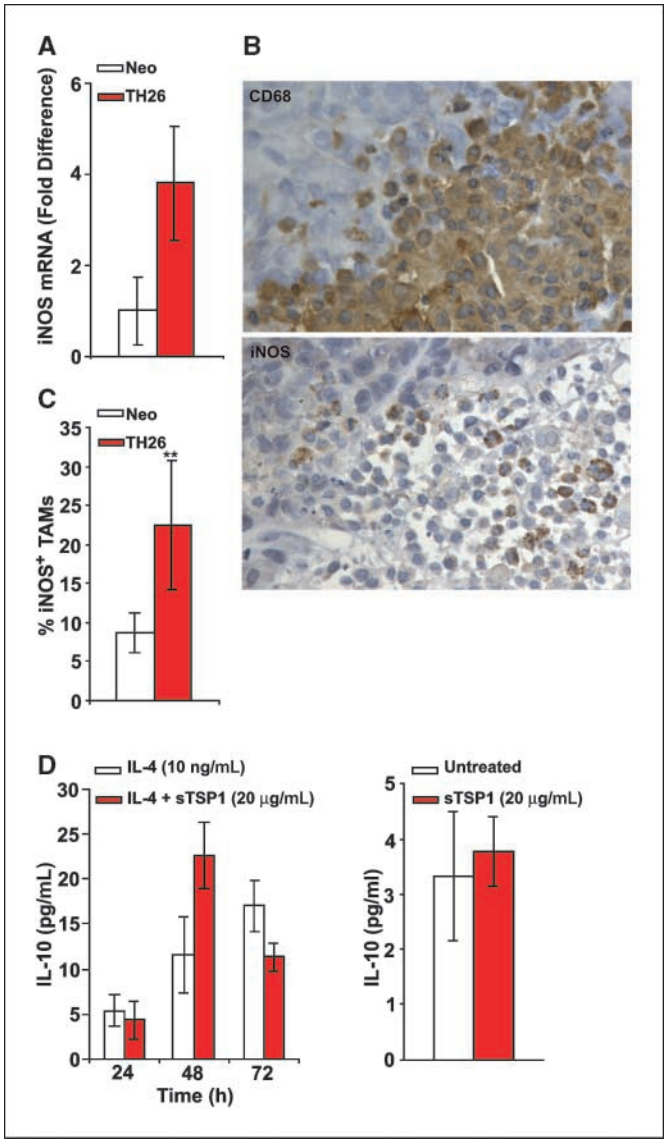

Figure 3. Increased iNOS-expressing M1 TAMs in TSP1-overexpressing tumors. *A,* real-time quantitative reverse transcription-PCR analysis of mRNA expression in TH26 and Neo tumors from six NIH-*nu/nu* mice. Fold difference was adjusted to HPRT1 internal control values. Relative quantification of iNOS was calculated by the $2^{-\Delta\Delta CT}$ method. *B,* immunohistochemical analysis of TH26 and Neo tumors in NIH-*nu/nu* mice. Adjacent sections were stained using rat anti-mouse CD68 antibody (clone FA-11; *top*) and rabbit polyclonal antibody to iNOS to detect M1-differentiated TAMs (*bottom*). The results are representative of tumor samples from 12 NIH-*nu/nu* mice, which showed identical patterns. Magnification, ×400. *C,* quantitative analysis of CD68-positive cells expressing iNOS in the tumor specimens was performed by evaluating the percentage of iNOS-positive macrophages in multiple 100× fields. The results are presented as the percentage of iNOS-positive TAMs in Neo control (*white column*) and TH26 tumors (*red column*). *D, left,* U937 cells (2×10^5/0.5 mL AIM-V) were incubated with IL-4 to induce M2 differentiation in the presence or absence of soluble TSP1. At the indicated times, the supernatants were harvested, and IL-10 was determined using a multiplex ELISA array as described in Materials and Methods. *Right,* U937 cells were differentiated with IL-4 for 72 h, and then the differentiated cells (1×10^6/1 mL AIM-V) were incubated with soluble TSP1. After 12 h of incubation, the supernatants were harvested, and IL-10 was determined using a multiplex ELISA array.

Increased M1 macrophage recruitment into TSP1-overexpressing tumors. Activated murine macrophages metabolize L-arginine via two main pathways that are catalyzed by the inducible enzymes iNOS and arginase. Increased iNOS is characteristic of M1 macrophages, and arginase-1 is a marker of M2 macrophages (38). To compare iNOS expression *in vivo*, total RNA extracted from six randomly selected Neo and TH26 tumors was analyzed using real-time PCR. A 3.8-fold increase in iNOS expression was found in TH26 tumors (Fig. 3*A*). In contrast, the M2 marker arginase-1 was equally expressed in both tumors (data not shown). Staining of tumor sections using an iNOS antibody showed an increased percentage of iNOS-expressing TAMs in TH26 versus Neo tumors ($P \leq 0.001$; Fig. 3*B* and *C*). Taken together, these data show that M1 cytotoxic macrophages are a minor fraction of the TAMs in MDA-MB-435 tumors, but their recruitment or differentiation is increased when the tumors express TSP1.

To address whether M2 macrophage differentiation or function is sensitive to TSP1 *in vitro*, U937 cells were differentiated using IL-4 for 72 h in the presence or absence of soluble TSP1. IL-10 levels in the cell culture supernatants, a marker of M2 polarization, showed no significant differences (Fig. 3*D, left*). Similarly, incubation of IL-4–differentiated U937 cells with soluble TSP1 (20 μg/mL) for 12 h did not alter IL-10 secretion (Fig. 3*D, right*).

TSP1 stimulates macrophage cytotoxicity toward breast carcinoma and melanoma cells. To determine whether TSP1 can regulate tumor cell killing by macrophages, we performed dynamic monitoring of macrophage-mediated cytolysis. IFN-γ–differentiated U937 cells were incubated with MDA-MB-231 breast carcinoma cells (at an E:T ratio of 40:1) in the RT-CES system. The cell index readout assesses changes in viable adherent cells

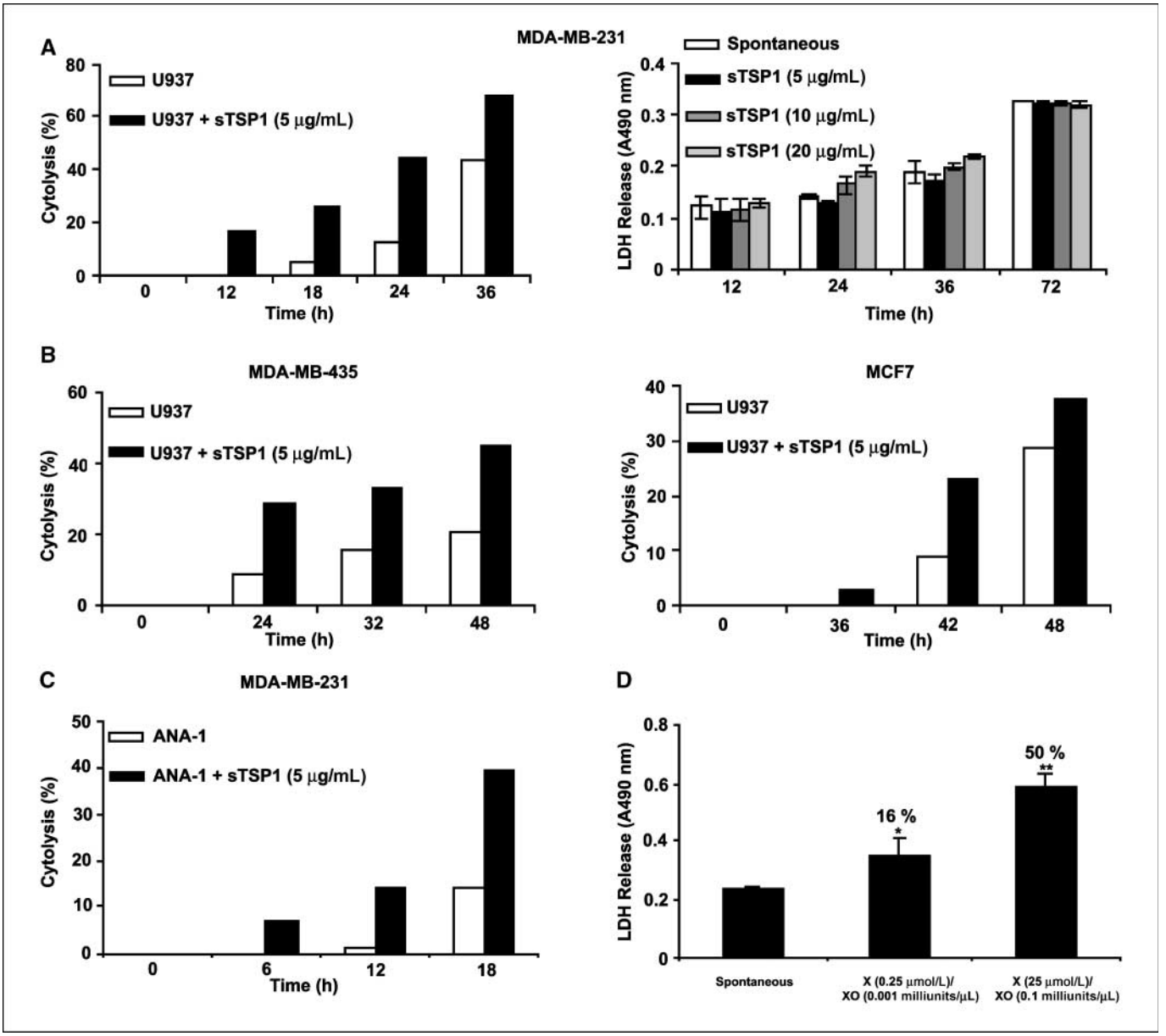

Figure 4. TSP1 increases U937 human monocytic cell–mediated tumoricidal activity. *A, left,* and *B,* MDA-MB-231, MDA-MB-435, and MCF-7 target cells (2×10^4/150 μL RPMI 1640 with 10% FBS) were seeded into 16-well sensor plates and incubated for up to 24 h. After this incubation, differentiated U937 effector cells (8×10^5/50 μL RPMI 1640 with 10% FBS) were directly added into wells containing target cells in the presence or absence of soluble TSP1. The measurements were automatically collected by the analyzer RT-CES system for up to 48 h. Data are representative of three different experiments. *A, right,* MDA-MB-231 cells (2×10^4/200 μL RPMI 1640 with 10% FBS) were seeded into 96-well plates and incubated for up to 72 h in the presence or absence of soluble TSP1 (5–20 μg/mL). At the indicated times, medium samples were collected and LDH released was measured as described in Materials and Methods. The absorbance was recorded at 490 nm. Data are representative of two different experiments. *C,* MDA-MB-231 target cells (2×10^4/150 μL RPMI 1640 with 10% FBS) were seeded into 16-well sensor plates and incubated for up to 24 h. After this incubation, activated ANA-1 effector cells (8×10^5/50 μL RPMI 1640 with 10% FBS and 0.5 mmol/L aminoguanidine) were directly added into wells containing target cells in the presence or absence of soluble TSP1. The measurements were automatically collected by the RT-CES system for up to 18 h. Data are representative of four different experiments. *D,* MDA-MB-231 target cells (2,500/100 μL RPMI 1640 with 1.25% FBS) were seeded into 96-well plates and incubated for 72 h in the presence or absence of different doses of xanthine (*X*) and xanthine oxidase (*XO*). The medium samples were collected, and LDH released was measured. The absorbance was recorded at 490 nm. The percentage of cytotoxicity is shown above each column and is calculated as (experimental − spontaneous/maximum − spontaneous) × 100. Maximum LDH release was determined by complete lysis of target cells.

by electrical impedance. Differentiated U937 cells expressed constitutive cytotoxic activity against MDA-MB-231 cells (Fig. 4*A*). Moreover, tumoricidal activity was increased 5-fold after 18 h of incubation with soluble TSP1 (5 μg/mL; Fig. 4*A, left*). This concentration of TSP1 did not show any direct cytotoxic activity against MDA-MB-231 cells (Fig. 4*A, right*). TSP1 similarly enhanced cytotoxicity against MDA-MB-435 melanoma and MCF-7 breast carcinoma cell targets (Fig. 4*B, left* and *right*, respectively). Because our *in vivo* model used a human tumor xenograft in mice, we also examined the mouse macrophage cell line ANA-1 as effector against human MDA-MB-231 target cells. Activated ANA-1 cells expressed constitutive cytotoxic activity against MDA-MB-231 cells in the presence of an iNOS inhibitor (0.5 mmol/L aminoguanidine) to permit O_2^- accumulation (39). A

10-fold increase in tumoricidal activity was recorded after 12 h of incubation with soluble TSP1 (5 μg/mL; Fig. 4*C*).

TSP1 has been shown to enhance chemoattractant fMLP-mediated superoxide generation in human neutrophils (14), suggesting that increased ROS mediates the tumoricidal activity of TSP1-stimulated cells. To confirm sensitivity to O_2^-, MDA-MB-231 cells were treated with xanthine and xanthine oxidase to generate O_2^-. Addition of O_2^- dose dependently increased LDH release from the cells (Fig. 4*D*).

TSP1 increases extracellular release of O_2^-. To determine whether the known activity of TSP1 to enhance O_2^- release in human neutrophils extends to M1-differentiated U937 cells, IFN-γ–differentiated U937 cells were stimulated with PMA (100 ng/mL), and O_2^- generation was assessed using luminol chemiluminescence. Incubation of differentiated U937 cells with soluble TSP1 (20 μg/mL) significantly increased ($P \leq 0.001$) PMA-mediated O_2^- production (Fig. 5*A, left*), and 25 units of SOD completely abolished this signal (data not shown), confirming that O_2^- was responsible for the chemiluminescent signal. This also indicates that TSP1 stimulates extracellular O_2^- production because SOD does not degrade intracellular O_2^-. Similar enhancement of O_2^- generation by TSP1 was observed in monocytes isolated from human PBMCs and in a murine macrophage cell line ANA-1 (Fig. 5*A, middle* and *right*). Trimeric recombinant constructs (residues 1–356) containing the N-modules of TSP1 (NoC1) and TSP2 (residues 1–359, NoC2) but not other recombinant regions of TSP1 enhanced O_2^- production (Fig. 5*B*).

The N-module of TSP1 interacts with several receptors, including $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_6\beta_1$ integrins (40). Because $\alpha_3\beta_1$ integrin recognizes the N-module of TSP1 but not TSP2 (41), we examined the role of $\alpha_4\beta_1$ and $\alpha_6\beta_1$ integrins. Differentiated U937 cells are known to express $\alpha_4\beta_1$ (42), and we found that they also express $\alpha_6\beta_1$ (Fig. 5*C*). The $\alpha_6\beta_1$-binding peptide LALERKDHSG (24) and a function-blocking $\alpha_6\beta_1$ antibody partially inhibited the activity of TSP1 on O_2^- generation. In contrast, the $\alpha_4\beta_1$ antagonist phLDVP had no effect. These results identify a specific requirement for $\alpha_6\beta_1$ integrin to mediate TSP1 binding to human monocytes and the subsequent activation of intracellular signaling pathways required for O_2^- production (Fig. 5*D*).

TSP1-stimulated O_2^- production in macrophages requires intracellular Ca^{2+}. Ligation of some integrins triggers a transient elevation in intracellular free Ca^{2+} (43, 44). Ca^{2+} is a second

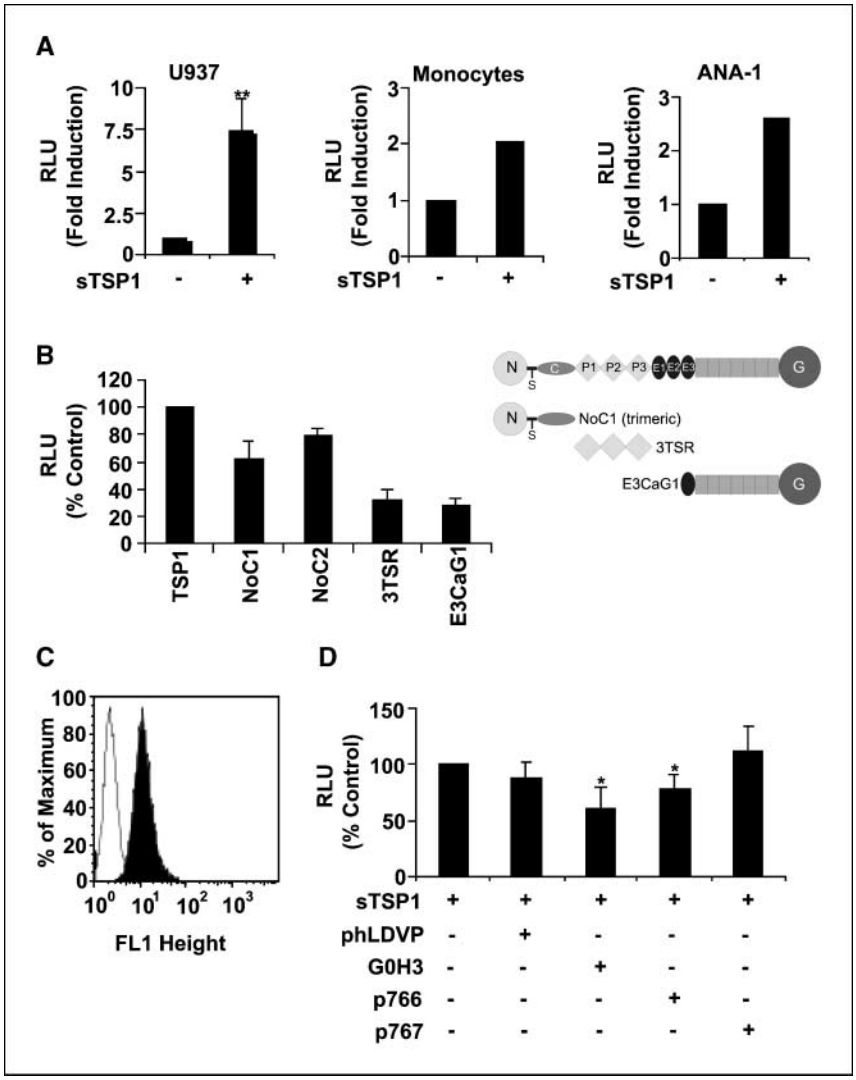

Figure 5. TSP1 enhances extracellular release of O_2^- in human and mouse monocytic cells. *A, left,* differentiated U937 cells (5×10^5 per condition) were stimulated with PMA (100 ng/mL) in the presence or absence of soluble TSP1 (20 μg/mL) for up to 70 min, and quantitative analysis of O_2^- levels was performed with a LumiMax Superoxide Anion Detection kit and quantified using a luminometer. The results are presented as the ratio maximum activity [relative luminescence units (*RLU*)] per minute. *Columns,* mean of five different experiments; *bars,* SD. *Middle,* analysis of O_2^- release from human monocytic cells. Monocytes obtained from human PBMCs (5×10^5 per condition) were stimulated with PMA (10 ng/mL) in the presence or absence of soluble TSP1 (20 μg/mL) for 30 min. The results are presented as the ratio maximum activity (RLU) per minute. Data are representative of four different experiments. *Right,* activated ANA-1 cells (8×10^5 per condition) were stimulated with PMA (100 ng/mL) and aminoguanidine (0.5 mmol/L) in the presence or absence of soluble TSP1 (5 μg/mL) for 130 min. The results are presented as RLU and are representative of two different experiments. *B,* analysis of O_2^- release from differentiated U937 cells (5×10^5 per condition) stimulated as described above in the presence or absence of soluble TSP1 (20 μg/mL), NoC1, 3TSR, or E3CaG1 (10 μg/mL) for up to 40 min. The results are presented as a percentage of control RLU determined in the presence of TSP1. *Columns,* mean of up to four different experiments; *bars,* SD. *C,* surface α_6 integrin expression in differentiated U937 cells was analyzed using flow cytometry as described in Materials and Methods. *D,* analysis of O_2^- release from differentiated U937 cells (5×10^5 per condition) stimulated as described above with soluble TSP1 (20 μg/mL) in the presence or absence of the $\alpha_4\beta_1$ integrin antagonist phLDVP (1 μmol/L), the function-blocking rat anti-human α_6 monoclonal antibody (clone G0H3; 5 μg/mL), p766, or control peptide (p767, 200 μmol/L) for up to 40 min. The results are presented as a percentage of control RLU determined in the presence of TSP1 alone. *Columns,* mean of up to five different experiments; *bars,* SD.

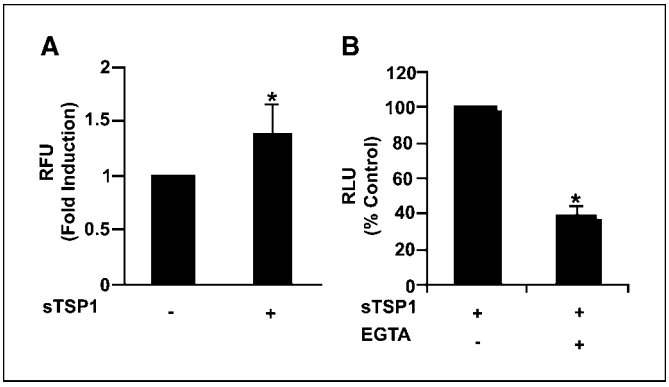

Figure 6. Ca^{2+} is an intracellular second messenger for activation of the oxidative burst in TSP1-stimulated U937 monocytic cells. *A,* analysis of intracellular free Ca^{2+} was performed in differentiated U937 cells. U937 cells (4×10^5 per condition) were loaded with Fluo-4 for 30 min and then treated with soluble TSP1 (20 μg/mL) as described in Materials and Methods. The cells were then placed in a fluorometer and measurements were acquired for 40 min. Results are presented as fold induction in TSP1-treated (+) relative to untreated cells (−). *Columns,* mean of four different experiments; *bars,* SD. *B,* analysis of O_2^- release from differentiated U937 cells (5×10^5 per condition) stimulated with PMA (100 ng/mL) and soluble TSP1 (20 μg/mL) in the presence or absence of EGTA (1 mmol/L) for up to 45 min. The levels of O_2^- were determined by luminescence as described in Fig. 5. The results are presented as a percentage of control RLU determined in the presence of TSP1 alone and are representative of two different experiments.

messenger for activation of NADPH oxidase in human monocytes (45). This result suggested that increased levels of Ca^{2+} might account for the enhancement by TSP1 of O_2^- production in differentiated U937 cells. Cells were loaded with Fluo-4 for 30 min and then treated with soluble TSP1 (20 μg/mL) for 25 to 50 min. Addition of TSP1 caused a significant rise in intracellular free Ca^{2+} (Fig. 6*A*). This increase was eliminated completely following chelation of extracellular Ca^{2+} by the addition of 1 mmol/L EGTA (data not shown). To further confirm the role of Ca^{2+} in respiratory burst activity, differentiated U937 cells were treated with EGTA before the addition of PMA and TSP1. As shown in Fig. 6*B*, chelation of extracellular Ca^{2+} significantly decreased the stimulatory effect of TSP1 on PMA-mediated O_2^- generation in differentiated U937 cells, suggesting that a Ca^{2+}-dependent mechanism is involved in TSP1 modulation of the macrophage respiratory burst.

Discussion

Several studies have suggested that TSP1 plays an important role in the recruitment of monocytes and macrophages to sites of tissue injury or inflammation (1, 12). Here, we provide evidence that overexpression of TSP1 in tumors increases macrophage recruitment *in vivo*. Tumor cells produce a range of chemotactic factors for macrophages. MCP-1 stimulates recruitment of macrophages into tumors *in vivo* (46), and decreased expression of these chemokines was reported to limit infiltration of macrophages into an excisional wound in TSP1-null mice (1). However, soluble TSP1 did not increase MCP-1 release from differentiated U937 human monocytic cells. PAI-1 is also required for cell migration *in vitro* (33), and evidence is emerging for a critical role of PAI-1 in macrophage migration *in vitro* and *in vivo* (32). TSP1 up-regulates PAI-1 in pancreatic cancer cells (47), and PAI-1 expression is up-regulated in TSP1-expressing MDA-MB-435 cells (34). We found that TSP1 acutely increased PAI-1 production by differentiated human and mouse macrophages, and strong TAM expression of PAI-1 was observed in TSP1-overexpressing tumors *in vivo*. This acute change in PAI-1 expression in response to TSP1 is at least partially TGFβ mediated. Activation of latent TGFβ is mediated by the type 1 repeats of TSP1, but we found no induction of PAI-1 by this domain. Therefore, TSP1 probably induces PAI-1 via bound active TGFβ rather than activation of latent TGFβ produced by U937 cells. Although additional cytokines may be involved, autocrine induction of PAI-1 via tumor cell produced TSP1 as well as paracrine induction of PAI-1 expression in TAMs could increase macrophage recruitment into the tumor.

Whether increased macrophage recruitment inhibits or enhances tumor growth depends on their differentiation state. The tumor environment can educate TAMs toward a tumor-promoting phenotype (M2; ref. 18), which may prevent further macrophage migration within the tumor and ensure constant production of growth and angiogenic factors. In addition to contributing to macrophage infiltration by stimulating PAI-1 signaling in TAMs, we found that TSP1 expression in the tumor selectively increases M1 macrophage infiltration. This may provide a selective pressure distinct from its antiangiogenic activity to account for the frequently observed down-regulation of TSP1 during tumor progression and its ability to inhibit tumor growth when reexpressed.

In general, M1 macrophages are efficient producers of reactive oxygen and nitrogen intermediates that mediate resistance against tumors (17). Here, we provide evidence that tumor expression of TSP1 increases M1 polarization of TAMs assessed by iNOS expression. TSP1 was previously shown to enhance cytokine-induced and chemoattractant fMLP-induced respiratory burst in human neutrophils (13, 14). We now show that TSP1 enhances PMA-mediated respiratory burst in U937 cells differentiated along an M1 pathway using IFN-γ. TSP1 stimulates the cytotoxic activity of differentiated human U937 cells and murine ANA-1 cells against several human breast carcinoma and melanoma cell lines. This contrasts with the reported activity of U937 cells to support tumor growth in an M2 manner when coinjected with prostate carcinoma cells (48). We found no effect of TSP1 on M2 differentiation of these cells *in vitro*. Therefore, U937 monocytes have the capacity to differentiate along both pathways, but TSP1 selectively enhances the cytotoxic effector function of M1 macrophages.

The NH_2-terminal domains of TSP1 and TSP2 are sufficient for this priming activity but not for PAI-1 induction. The NH_2-terminal domain of TSP1 mediates interactions with several integrin and nonintegrin receptors (40). Differentiated U937 cells express $\alpha_4\beta_1$ (42) and $\alpha_6\beta_1$ integrins, and inhibition of $\alpha_6\beta_1$ using a TSP1 peptide or a function-blocking $\alpha_6\beta_1$ antibody provides evidence that this integrin mediates intracellular signaling pathways leading to increased O_2^- production.

Interactions between integrins and their ligands can trigger transient elevation in intracellular free Ca^{2+} (43, 44), and Ca^{2+} is a well-known intracellular second messenger for signaling the generation of O_2^- in human monocytes (45). We found that addition of TSP1 to differentiated U937 cells caused a significant increase in intracellular free Ca^{2+} and that chelation of extracellular Ca^{2+} inhibits the stimulatory effect of TSP1 on mobilization of intracellular Ca^{2+} and PMA-mediated O_2^- generation in differentiated U937 cells. Taken together, these data suggest that a Ca^{2+}-dependent mechanism is involved in TSP1 modulation of the

macrophage respiratory burst. However, we cannot exclude the possibility that additional receptors recognized by the N-domains of TSP1 might contribute to O_2^- production by macrophages. It is interesting that the NH_2-terminal region of TSP2 shares this activity to stimulate O_2^- production by macrophages. Loss of TSP2 has also been noted with progression in some cancers, and overexpression of TSP2 limits tumor growth in murine models (19). Our data suggest that this may involve both suppression of angiogenesis and enhancement of innate antitumor immunity.

Soluble TSP1 at 5 μg/mL was sufficient to increase U937-mediated cytotoxicity in all tumor cell targets tested. TSP1 was also reported to directly induce death of MDA-MB-231 and MCF-7 cells when used at 10 μg/mL for 24 h (49). Our results using a cytotoxicity assay based on LDH release showed minimal cytotoxic activity of soluble TSP1 against MDA-MB-231 cells even after 72 h of incubation. Our data indicate that TSP1 stimulates macrophage-mediated tumor cell death due to accumulation of ROS. Physiologic doses of O_2^- generated using xanthine/xanthine oxidase were sufficient to inhibit MDA-MB-231 breast carcinoma cell viability.

In conclusion, the data presented here show that stimulation of M1-differentiated human monocytic cells with TSP1 enhances tumor cell killing *in vitro* via production of reactive oxygen intermediates. *In vivo*, TSP1 promotes M1 macrophage recruitment into tumors while decreasing tumor growth. Clearly, TSP1 can also inhibit tumor growth via its antiangiogenic activity, but our results suggest that TSP1 plays an additional role in tumor immunity by increasing M1 macrophage recruitment and cytotoxicity. Avoiding this innate immune surveillance could provide a second selective pressure to reduce TSP1 expression during tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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B.ADDITIONAL RESULTS

Elevated production of proinflammatory cytokines and chemokines by TSP1-activated human monocytic cells.

TSP1 is secreted at sites of injury and inflammation by monocytes (1), and differentially regulates the release of IL-6 and IL-10 by U937 human monocytic cells (2). However, the underlying mechanism for regulation of cytokine expression has not been delineated. To determine the transcriptional events associated with TSP1 regulation of cytokine and chemokine expression we performed real-time quantitative reverse transcription-PCR analysis of IFNγ-differentiated U937 cells treated for 12 h with soluble TSP1. Soluble TSP1 treatment resulted in a significant increase or decrease in RNA levels of 31 key genes involved in the inflammatory response (**Table I**).

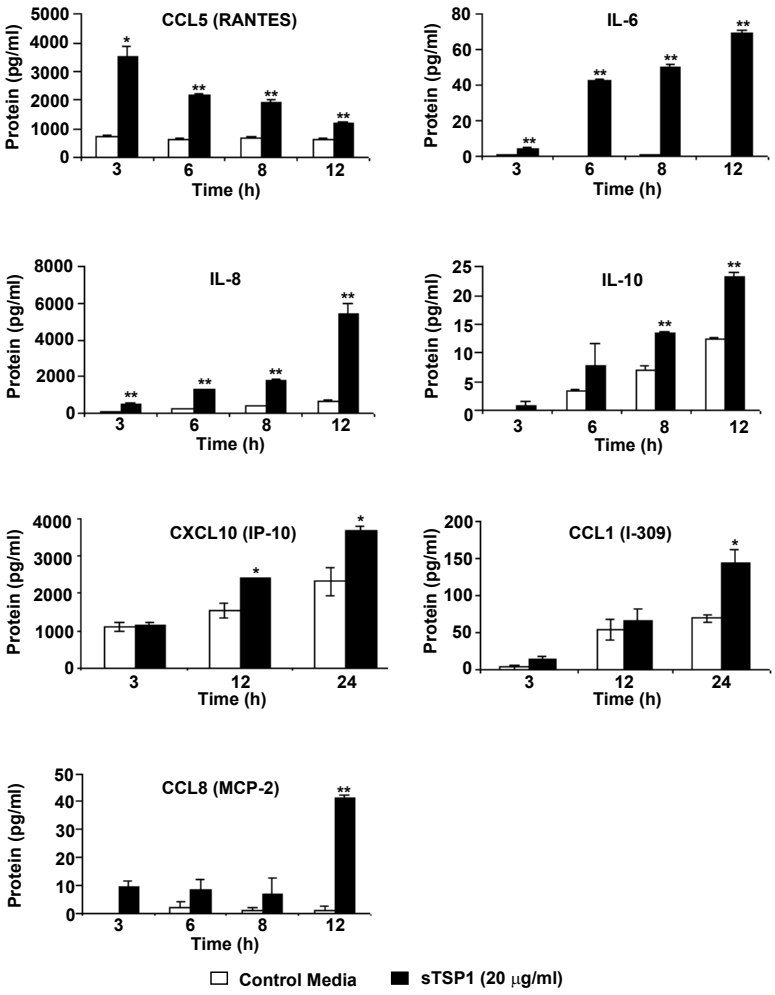
Table I. Genes differentially expressed in TSP1-activated human monocytic cells[a].

UniGene	GenBank	Gene Name	Gene Symbol	Fold Up- or Down-regulation
Hs.529053	NM_000064	Complement component 3	C3	7.70
Hs.72918	NM_002981	Chemokine (C-C motif) ligand 1 (I-309)	CCL1	4.23
Hs.414629	NM_005408	Chemokine (C-C motif) ligand 13 (MCP-4)	CCL13	7.56
Hs.143961	NM_002988	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated) (DC-CK1)	CCL18	2.05
Hs.303649	NM_002982	Chemokine (C-C motif) ligand 2 (MCP-1)	CCL2	3.51
Hs.75498	NM_004591	Chemokine (C-C motif) ligand 20 (MIP-3α)	CCL20	15.33
Hs.310511	NM_005624	Chemokine (C-C motif) ligand 25 (TECK)	CCL25	-3.18
Hs.514107	NM_002983	Chemokine (C-C motif) ligand 3 (MIP-1α)	CCL3	13201.95
Hs.75703	NM_002984	Chemokine (C-C motif) ligand 4 (MIP-1β)	CCL4	20561.73
Hs.251526	NM_006273	Chemokine (C-C motif) ligand 7 (MCP-3)	CCL7	3.27
Hs.652137	NM_005623	Chemokine (C-C motif) ligand 8 (MCP-2)	CCL8	63.52
Hs.644637	NM_000648	Chemokine (C-C motif) receptor 2	CCR2	-9.04
Hs.113222	NM_005201	Chemokine (C-C motif) receptor 8	CCR8	-3.22
Hs.225946	NM_006641	Chemokine (C-C motif) receptor 9	CCR9	-2.05
Hs.789	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (Gro-α)	CXCL1	3.75
Hs.632586	NM_001565	Chemokine (C-X-C motif) ligand 10 (IP-10)	CXCL10	14.35
Hs.632592	NM_005409	Chemokine (C-X-C motif) ligand 11 (I-TAC)	CXCL11	6.00
Hs.100431	NM_006419	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant) (BCA-1)	CXCL13	2.85
Hs.590921	NM_002089	Chemokine (C-X-C motif) ligand 2 (Gro-β)	CXCL2	6.15
Hs.89690	NM_002090	Chemokine (C-X-C motif) ligand 3 (Gro-γ)	CXCL3	75.97
Hs.89714	NM_002994	Chemokine (C-X-C motif) ligand 5 (ENA-78)	CXCL5	3.61
Hs.164021	NM_002993	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) (GCP-2)	CXCL6	3.45
Hs.211575	NM_000605	Interferon, alpha 2	IFNA2	6.15
Hs.193717	NM_000572	Interleukin 10	IL-10	75.97
Hs.126256	NM_000576	Interleukin 1, beta	IL-1B	3.55
Hs.166371	NM_173205	Interleukin 1 family, member 7 (zeta)	IL-1F7	-2.07
Hs.624	NM_000584	Interleukin 8	IL-8	8.02
Hs.960	NM_000590	Interleukin 9	IL-9	3.28
Hs.406228	NM_002186	Interleukin 9 receptor	IL-9R	-2.12
Hs.376208	NM_002341	Lymphotoxin beta (TNF superfamily, member 3)	LTB	-2.06
Hs.313	NM_000582	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	4.04

[a] The table shows a selection of key genes involved in the inflammatory response. In each row, genes show their fold difference between IFNγ-differentiated U937 cells untreated or treated with soluble TSP1 (20 μg/ml) for 12 h.

To substantiate the response of IFNγ-differentiated U937 cells to soluble TSP1 treatment, we further evaluated the protein level of some of these target genes by performing Multiplexed ELISA to detect secretion of CCL5 (RANTES), IL-6, IL-8, IL-10, CXCL10 (IP-10), CCL1 (I-309), and CCL8 (MCP-2) into cell culture medium by differentiated U937 cells upon TSP1 treatment. Consistent with results at the transcriptional level, these secreted proteins were also elevated in supernatants collected from differentiated U937 cells after TSP1 treatment (**Fig. 1**). Incubation of differentiated U937 cells with TSP1 resulted in a time-dependent increase in RANTES expression, with a maximal 4.8-fold induction at 3 h (**Fig. 1**, $p<0.05$). Incubation of differentiated U937 cells with TSP1 resulted in an acute time-dependent increase in IL-6, IL-8, and MCP-2 expression, with a maximal 89.5-, 8.4-, and 39.4-fold induction at 8, and 12 h, respectively (**Fig. 1**, $p\leq0.001$). As shown in **Fig. 1**, TSP1 treatment also led to a (1.6-2)-fold increase in IL-10, IP-10, and I-309 expression in culture supernatant.

Figure 1. Elevated production of proinflammatory cytokines and chemokines by TSP1-activated human monocytic cells.



IFNγ-differentiated U937 cells (1 x 10^6/0.5 ml RPMI 1640 with 0.5% FBS) were incubated for 3, 6, 8, 12, and 24 h in the absence or the presence of soluble TSP1 (20 μg/ml). CCL5 (RANTES), IL-6, IL-8, IL-10, CXCL10 (IP-10), CCL1 (I-309), and CCL8 (MCP-2) levels in the culture supernatant were determined using a multiplexed ELISA array (Quansys Biosciences), as described in *Materials* and *Methods*. Data are representative of at least four different experiments.

Because the N-terminal module of TSP1 increases FMLP-mediated O_2^- generation and chemotaxis by human neutrophils (3, 4), and PMA-mediated O_2^- generation by IFNγ-differentiated U937 human monocytic cells (5), we examined the role of the N-terminal module of TSP1 in mediating cytokine and chemokine expression. Using an anti-TSP monoclonal antibody against the N-terminal module of TSP1, designated A2.5 (6), we found that incubation of IFNγ-differentiated U937 cells with soluble TSP1 (20 μg/ml) in the presence of the monoclonal antibody A2.5 (10 μg/ml) did not alter RANTES and I-309 secretion (**Fig. 2***A*). Because different modules of TSP1 often mediate opposite effects, we examined the role of the C-terminal domain of TSP1 in mediating cytokine and chemokine expression. We used an anti-TSP monoclonal antibody against the C-terminal module of TSP1, designated C6.7 (6, 7), and we found that incubation of IFNγ-differentiated U937 cells with soluble TSP1 (20 μg/ml) in the presence of the monoclonal antibody C6.7 (10 μg/ml) resulted in a time-dependent increase in RANTES expression (**Fig. 2***A left, p<0.001*), suggesting that the C-terminal domain of TSP1 can mediate inhibition of RANTES expression at the late time points of 12 and 24 h.

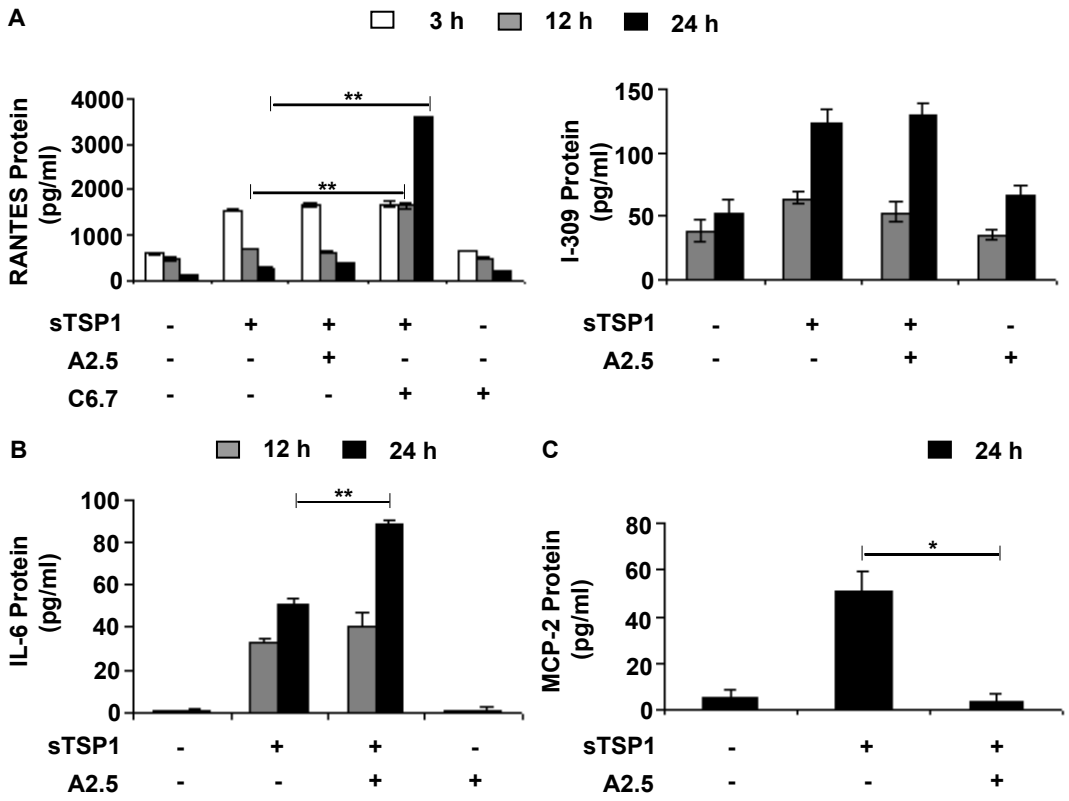
Alternatively, the antibody may increase and prolong a stimulation mediated by a different domain of TSP1 by cross-linking TSP1.

In addition, we found that 24 h incubation of IFNγ-differentiated U937 cells with soluble TSP1 (20 μg/ml) in the presence of the monoclonal antibody A2.5 (10 μg/ml) resulted in a significant increase in IL-6 expression (**Fig. 2***B, p<0.001*). Moreover, incubation of IFNγ-differentiated U937 cells with soluble TSP1 in the presence of the monoclonal antibody A2.5 resulted in an increase in IL-8, IL-10, and IP-10 expression (*Data not shown*). Our data suggest that the N-terminal domain of TSP1 can mediate inhibition of IL-6, IL-8, IL-10, and IP-10 expression at the late time points of 12 and 24 h. Alternatively, these increased responses may also result from cross-linking of TSP1 by the antibody.

On the contrary, we found that 24 h incubation of IFNγ-differentiated U937 cells with soluble TSP1 (20 μg/ml) in the presence of the monoclonal antibody A2.5 (10 μg/ml) resulted in a significant decrease in MCP-2 expression (**Fig. 2***C, p<0.05*), indicating that the N-terminal domain of TSP1 can mediate stimulation of MCP-2 expression, which is blocked by this antibody.

All this complexity is due to the conformational flexibility of TSP1, and the distinct expression profiles and activity status of the TSP1 receptors in the cells, and it makes it difficult to reconcile apparently contradictory results.

Figure 2. Role of the N-terminal domain of TSP1 in mediating cytokine and chemokine expression

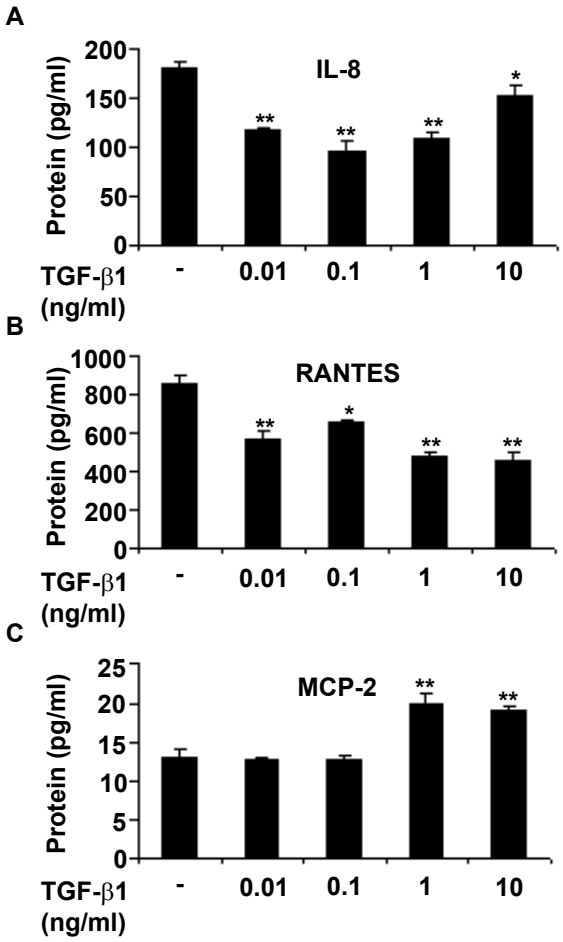


A-C, IFNγ-differentiated U937 cells (1 x 10^6/0.5 ml RPMI 1640 with 0.5% FBS) were incubated for 3, 12, and 24 h in the absence or the presence of soluble TSP1 (20 μg/ml), and anti-TSP monoclonal antibodies A2.5 and C6.7 (10 μg/ml). CCL5 (RANTES), CCL1 (I-309), IL-6, IL-8, IL-10, CXCL10 (IP-10), and CCL8 (MCP-2) levels in the culture supernatant were determined using a multiplexed ELISA array (Quansys Biosciences), as described in *Materials* and *Methods*. Data are representative of two different experiments.

Role of TGF-β1 in TSP1-mediated cytokine and chemokine expression by human monocytic cells.

PAI-1 is a TGF-β-inducible gene (8), and bioactive TGF-β present in platelet TSP1 (9) at least partially, mediates the stimulation of PAI-1 production by U937 cells (5). To determine whether TGF-β contributed to cytokine and chemokine responses in U937 cells, we first employed recombinant human TGF-β1, and we found that 12 h incubation of IFNγ-differentiated U937 cells with different doses of TGF-β1 resulted in a significant decrease in IL-8 and RANTES expression (**Fig. 3***A-B*). In addition, we found that 12 h incubation of IFNγ-differentiated U937 cells with 1 ng/ml of TGF-β1 resulted in a significant increase in MCP-2 expression (**Fig. 3***C, p<0.001*).

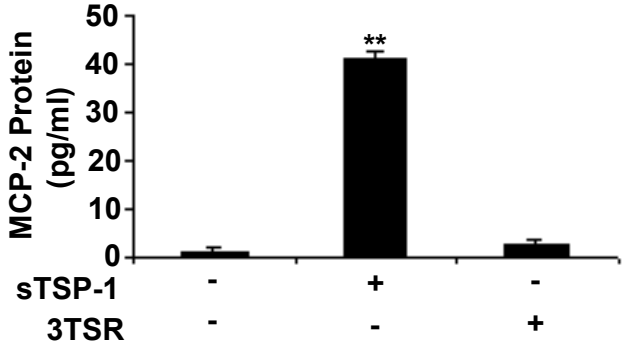
Figure 3. Role of TGF-β1 in cytokine and chemokine expression by human monocytic cells



A-C, IFNγ-differentiated U937 cells (1 x 10^6/0.5 ml RPMI 1640 with 0.5% FBS) were incubated for 12 h in the absence or the presence of different doses of recombinant human TGF-β1. IL-8, CCL5 (RANTES), and CCL8 (MCP-2) levels in the culture supernatant were determined using a multiplexed ELISA array (Quansys Biosciences), as described in *Materials* and *Methods*.

To determine whether TSP1-mediated activation of latent TGF-β (10) plays a role in MCP-2 release from differentiated U937 cells, we first examined the ability of recombinant 3TSR, the type 1 repeat domain of TSP1 responsible for the TSP1-mediated activation of latent TGF-β (11), to induce MCP-2 expression. The total amount of MCP-2 released from differentiated U937 cells did not change after treatment with 3 μg/ml of soluble recombinant 3TSR (**Fig. 4**). Therefore, activation of latent TGF-β probably does not account for the effect of TSP1 on total MCP-2 expression.

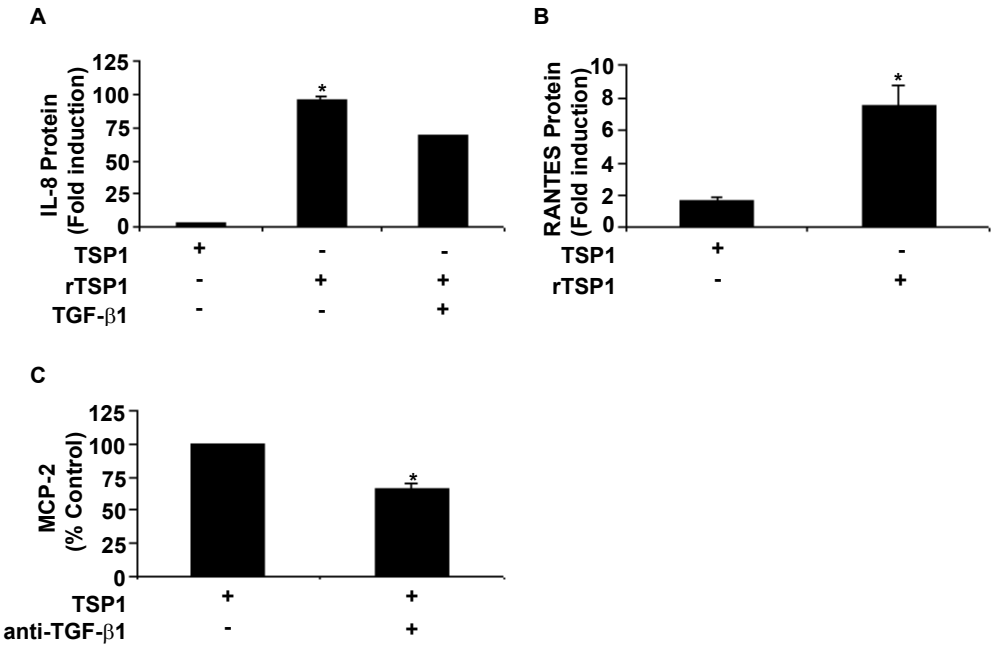
Figure 4. Role of TSP1-mediated activation of latent TGF-β1 in MCP-2 expression by human monocytic cells



IFNγ-differentiated U937 cells (1 x 10^6/0.5 ml RPMI 1640 with 0.5% FBS) were incubated for 12 h in the absence or the presence of soluble TSP1 (20 μg/ml), or soluble recombinant type 1 repeats of TSP1 (3TSR) (3 μg/ml). CCL8 (MCP-2) levels in the culture supernatant were determined using a multiplexed ELISA array (Quansys Biosciences), as described in *Materials* and *Methods*.

To test the hypothesis that bioactive TGF-β present in platelet TSP1 (9) regulates the cytokine and chemokine response in U937 cells, TSP1 stimulation of IL-8, RANTES, and MCP-2 production was then examined in the presence of recombinant human TSP1, which should lack TGF-β contamination, and a neutralizing TGF-β1 antibody (**Fig. 5**). Incubation of differentiated U937 cells with recombinant human TSP1 (20 μg/ml) resulted in a significant increase in IL-8 and RANTES expression, compared with platelet TSP1 (**Fig. 5***A, B, respectively, p<0.05*). Furthermore, recombinant human TSP1 at the same concentration in the presence of 1 ng/ml of recombinant TGFβ1, showed less stimulatory activity than recombinant TSP1 alone (**Fig. 5***A*). These results are consistent with our early observation that IL-8 and RANTES expression is inhibited by TGF-β1 in human monocytic cells (**Fig. 3**). In addition, TSP1 stimulation of MCP-2 production was examined in the presence of a neutralizing TGF-β1 antibody. At 5 μg/ml the neutralizing antibody partially inhibited TSP1-stimulated MCP-2 production (Fig. **5***C, p<0.05*). This result is consistent with our early observation that MCP-2 expression is enhanced by TGF-β1 in human monocytic cells (**Fig. 3**). Therefore, bioactive TGF-β present in platelet TSP1 at least partially mediates the stimulation of MCP-2 production by TSP1, but TSP1 lacking TGF-β is also active.

Figure 5. Effects of TGF-β1 on TSP1-mediated cytokine and chemokine expression in differentiated U937 human monocytic cells

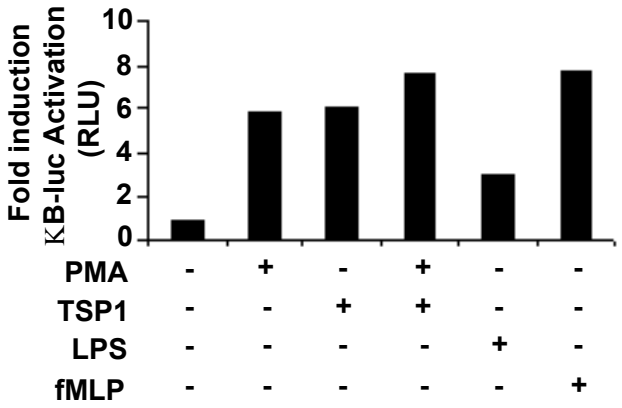


A-B, differentiated U937 cells (1 x 10^6/0.5 ml RPMI 1640 with 0.5% FBS) were incubated in the absence or the presence of soluble TSP1 (20 µg/ml), soluble recombinant human TSP1 (20 µg/ml), and recombinant human TGF-β1 (1 ng/ml). After 12 h incubation the supernatants were harvested, and total IL-8, and RANTES were determined using a multiplexed ELISA array (Quansys Biosciences), as described in *Materials* and *Methods*. Data represent the average and the standard deviation of two independent experiments. *C*, soluble TSP1 (20 µg/ml) was pre-incubated with neutralizing TGF-β1 antibody (*clone 9016*) for 45-60 min before addition to the differentiated U937 cells. Culture supernatants collected after 12 h were used to measure total MCP-2.

Identification of NF-κB as a downstream effector of TSP1-mediated cytokine and chemokine expression.

It is well established that NF-κB plays a role in the regulation of a variety of proinflammatory cytokines and chemokines. To address whether the increased production of proinflammatory cytokines and chemokines was caused by activation of NF-κB in TSP1-stimulated U937 human monocytic cells, we transfected IFNγ-differentiated U937 cells with a luciferase reporter driven by five copies of the NF-κB binding sites. 2 days after transfection, the cells were treated for 90 min with various stimuli. Incubation with PMA (100 ng/ml) or soluble TSP1 (20 μg/ml) caused a 6-fold increase in the NF-κB-mediated gene activation. In addition, incubation of differentiated U937 cells with soluble TSP1 increased PMA-mediated NF-κB activation (**Fig. 6**). Therefore, the increased production of some of the proinflammatory cytokines and chemokines is likely due to enhanced NF-κB signaling pathway after TSP1 stimulation in IFNγ-differentiated U937 human monocytic cells.

Figure 6. Increased NF-κB activity in TSP1-stimulated human monocytic cells



Differentiated U937 cells were co-transfected with pNF-κB-luc or pCIS-CK-luc (negative control), and phRL-*Renilla*. The cells were treated with PMA (100 ng/ml), soluble TSP1 (20 µg/ml), LPS (100 ng/ml), or fMLP (10^{-5}M) for 90 min. Cell lysates were collected and normalized to *Renilla*. Data shown are representative of two different experiments.

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IV. DISCUSIÓN

IV

A.DISCUSIÓN

Varios estudios indican que la TSP1 juega un papel importante en el reclutamiento de monocitos y de macrófagos en tejidos que han sufrido algún tipo de daño y en focos inflamatorios (1, 2). En este trabajo aportamos evidencias de que la sobreexpresión de TSP1 en tumores aumenta el reclutamiento de macrófagos *in vivo*. Las células tumorales producen una amplia variedad de factores quimiotácticos para los macrófagos. *In vivo*, la proteína-1 quimiotáctica para monocitos (MCP-1) estimula el reclutamiento de macrófagos al tumor (3). La disminución en la expresión de esta quimioquina también limita la infiltración de macrófagos en *excisional wounds* en ratones nulos para la TSP1 (1). No obstante, la incubación con TSP1 soluble de la línea celular monocítica humana U937 diferenciada con IFN-γ, no altera la secreción de la MCP-1.

In vitro, el inhibidor-1 del activador de plasminógeno (PAI-1) es necesario para la migración celular (4) y recientes estudios proponen es un factor crítico para la migración de los macrófagos tanto *in vitro* como *in vivo* (5). En células de cáncer de páncreas, la TSP1 es capaz de estimular la secreción del PAI-1 (6), así como en la línea celular MDA-MB-435 que sobreexpresa TSP1 (7). En nuestras manos, monocitos humanos diferenciados y macrófagos de ratón, responden al tratamiento con TSP1 con un aumento significativo de la secreción del PAI-1. *In vivo*, los TAMs procedentes de tumores que sobreexpresan TSP1 producen cantidades elevadas del PAI-1. Este cambio dramático en la expresión del PAI-1 en respuesta a la TSP1 está mediado, al menos en parte, por el TGF-β. Las repeticiones de tipo 1 de la TSP1 tienen la capacidad de interaccionar con el TGF-β latente y promover su activación. No obstante, este dominio de la TSP1 no presenta actividad ninguna en relación a la inducción del PAI-1. Por lo tanto, la TSP1 probablemente induce la secreción del PAI-1 a través del TGF-β activo que lleva unido, y no a través de la activación del TGF-β latente producido por las células U937.

Aunque no descartamos la posibilidad de que otras citoquinas puedan estar implicadas, la inducción autocrina del PAI-1 por parte de la TSP1 procedente de la célula tumoral, así como la inducción paracrina del PAI-1 en los TAMs, puede ser responsable del aumento en el infiltrado de macrófagos en el tumor.

El impacto que el aumento en el reclutamiento de macrófagos pueda tener en el crecimiento o disminución del tumor está en función del grado de diferenciación de estos macrófagos. El ambiente del tumor tiende a educar a los TAMs hacia un fenotipo promotor del tumor (8) y así, previene la migración de más macrófagos y se asegura la producción constante de factores de crecimiento y angiogénicos. En este estudio hemos descubierto que la expresión de TSP1 en el tumor, no sólo induce la migración de macrófagos estimulando la señalización vía PAI-1 en los TAMs, si no que favorece la migración selectiva de macrófagos de fenotipo M1. Esto podría favorecer el proceso de selección natural y, junto con su actividad anti-angiogénica, explicaría la represión en la expresión de la TSP1 que a menudo tiene lugar durante la progresión tumoral, así como la capacidad de esta proteína de inhibir el crecimiento tumoral cuando se reexpresa.

En general, los macrófagos con fenotipo M1 son productores eficaces de intermediarios de oxígeno y de nitrógeno, que median los procesos de resistencia contra el tumor (9). En este estudio, la detección de la enzima óxido nítrico sintetasa inducible (iNOS) pone de manifiesto que la expresión de la TSP1 en el tumor induce la polarización de los TAMs hacia un fenotipo M1.

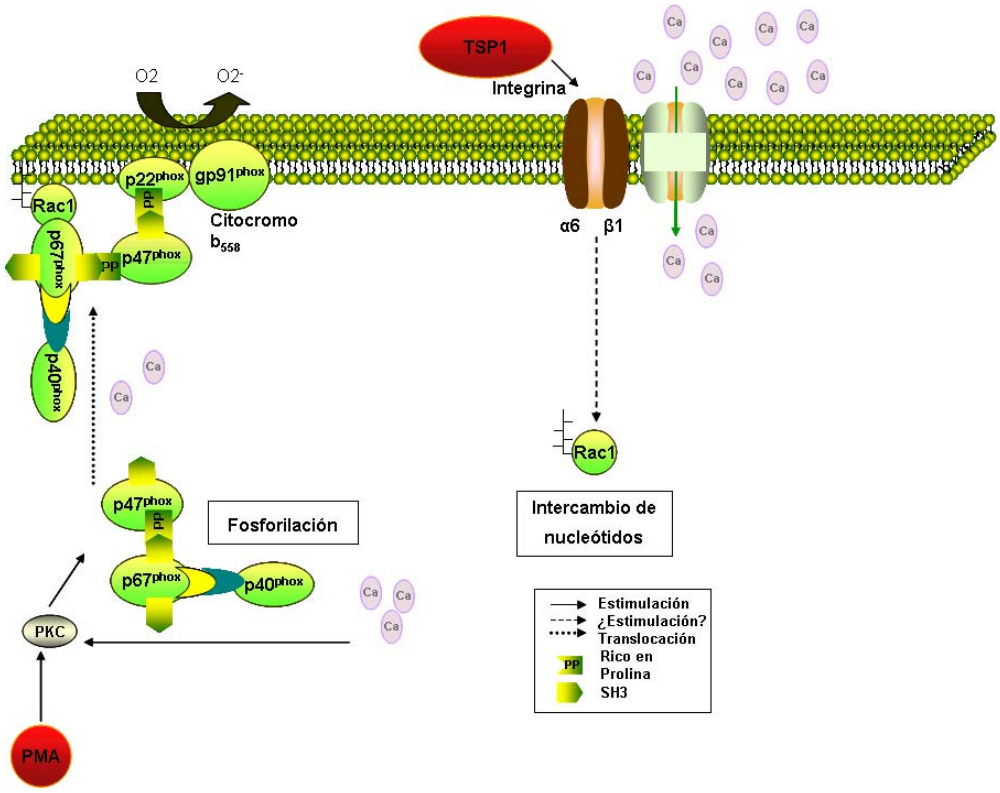
Estudios previos han demostrado que la TSP1 es capaz de potenciar el *respiratory burst* inducido por citoquinas y por el factor quimiotáctico fMLP en neutrófilos humanos (10, 11). En el presente trabajo de investigación demostramos que la TSP1 potencia el *respiratory burst* inducido por PMA en la línea celular U937 diferenciada hacia un fenotipo M1 usando IFN-γ. La TSP1 estimula la actividad citotóxica de las células U937 diferenciadas y de la línea celular de macrófagos de ratón ANA-1, para con distintas líneas celulares de cáncer de mama y de melanoma.

Por el contrario, las células U937 adquieren un fenotipo M2 promotor del crecimiento tumoral cuando se inyectan junto con células de cáncer de próstata (12). *In vitro*, no observamos efecto ninguno de la TSP1 en la diferenciación de tipo M2 de estas células. Por lo tanto, aunque los monocitos U937 parecen tener la capacidad de diferenciarse en ambos sentidos, la TSP1 estimula específicamente la función efectora citotóxica de los macrófagos M1.

Los dominios N-terminal de las TSP1 y 2 son suficientes para estimular el *respiratory burst* pero no para inducir la secreción del PAI-1. El dominio N-terminal de la TSP1 media la interacción con distintos receptores de tipo integrina y no-integrina (13). Las células U937 diferenciadas expresan las integrinas de tipo α4β1 (14) y α6β1. La inhibición de la integrina α6β1, usando anticuerpos que bloquean su función o péptidos de la TSP1, pone de manifiesto que a través de esta integrina se produce la activación de los mecanismos de señalización intracelular necesarios para la estimulación de la producción de O_2^-.

Las interacciones entre integrinas y sus ligandos pueden promover una elevación transitoria en los niveles intracelulares de Ca^{2+} libre (15, 16). Está ampliamente aceptado el papel del Ca^{2+} como segundo mensajero intracelular en la señalización que conduce a la generación de O_2^- en monocitos humanos (17). En este trabajo demostramos por un lado, que la incubación con TSP1 de células U937 diferenciadas hacia un fenotipo M1 se traduce en un aumento significativo en los niveles intracelulares de Ca^{2+} libre y por otro, que quelando el Ca^{2+} extracelular inhibimos el efecto estimulador de la TSP1 en la movilización del Ca^{2+} intracelular y la producción de O_2^- en células U937 diferenciadas y estimuladas con PMA (**Modelo I**).

Modelo I. La Trombospondina-1 induce la producción de O_2^- extracelular



En resumen, estos datos sugieren que un mecanismo dependiente de Ca^{2+} está implicado en la modulación del *respiratory burst* en macrófagos por la TSP1. No obstante, no podemos excluir la posibilidad de que otros receptores adicionales reconocidos por el dominio N-terminal de la TSP1 estén involucrados en la regulación de la producción de O_2^- en los macrófagos.

Resulta interesante que la región N-terminal de la TSP2 comparta esta actividad estimuladora de la producción de O_2^- en macrófagos. La pérdida de la TSP2 también está asociada a la progresión de algunos cánceres y su sobreexpresión limita el crecimiento tumoral en modelos experimentales en ratón (18). Los datos presentados en este estudio, sugieren que esta pérdida puede estar relacionada tanto con la supresión de la angiogénesis como con la potenciación de la respuesta inmune innata anti-tumoral.

La TSP1 en solución a una concentración de 5 µg/ml es suficiente para aumentantar la citotoxicidad de las células U937 contra todas las líneas celulares tumorales empleadas. La TSP1 puede inducir directamente la muerte celular en las líneas celulares de cáncer de mama MDA-MB-231 y MCF-7 cuando se usa en concentraciones de 10 µg/ml durante 24 h (19). Los resultados obtenidos de los ensayos de citotoxicidad basados en la liberación de la lactato deshidrogenada (LDH) muestran una actividad citotóxica mínima de la TSP1 en solución para con la línea celular MDA-MB-231, incluso después de 72 h de incubación.

Los datos presentados en este trabajo indican que el mecanismo por el que la TSP1 estimula la muerte de la célula tumoral mediada por macrófagos está relacionado con la acumulación de ROS. Así pues, la incubación de la línea celular de cáncer de mama MDA-MB-231 con dosis fisiológicas de O_2^-, fruto de la reacción enzimática de la xantina/xantina oxidasa, resulta suficiente para inhibir la viabilidad de estas células.

Los macrófagos activados y diferenciados de forma clásica como M1 no son sólo productores eficaces de intermediarios de oxígeno y de nitrógeno, si no que también lo son de citoquinas inflamatorias (9). Estudios previos han demostrado que la TSP1 es capaz de regular la producción de las IL-6 y -10 procedentes de células U937 estimuladas con PMA y lipopolisacárido (LPS) (20). En este trabajo de investigación demostramos que la TSP1 en solución aumenta la producción de citoquinas y quimioquinas proinflamatorias en las células U937 diferenciadas a macrófagos M1 usando IFN-γ, e identificamos al factor de transcripción NF-κB como un efector en esta ruta de señalización.

La TSP1 estimula la secreción de la IL-8 y de RANTES, quimioquinas relacionadas con el reclutamiento de neutrófilos y de leucocitos respectivamente, procedente de las células U937 diferenciadas con IFN-γ. Usando el TGF-β1 y la TSP1 recombiantes demostramos que el TGF-β1 bioactivo presente en la TSP1 procedente de plaquetas (21) regula negativamente la expresión de estas citoquinas.

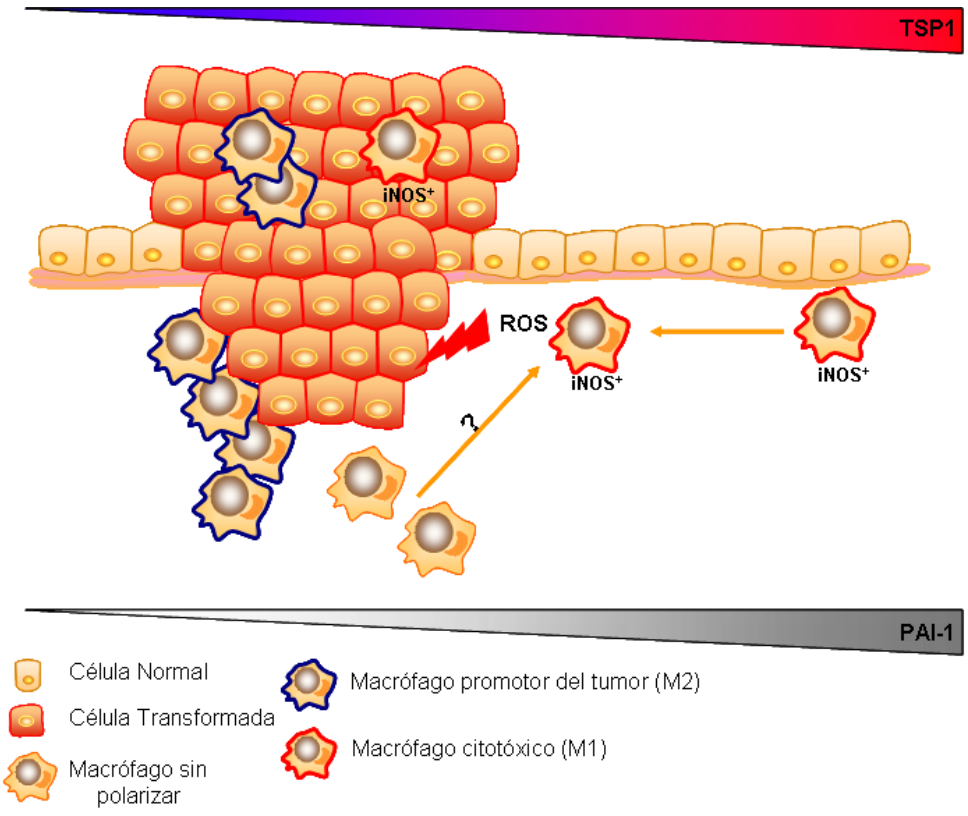
Los datos presentados en este trabajo indican que la TSP1 induce significativamente la producción de la MCP-2 por parte de macrófagos humanos diferenciados. Este cambio significativo en la expresión de la MCP-2 en respuesta a la TSP1 está mediado, al menos en parte, por el TGF-β. Las repeticiones de tipo 1 de la TSP1 llevan a cabo la activación del TGF-β latente, pero este dominio no induce la expresión de la MCP-2. Por lo tanto, la TSP1 probablemente induce la secreción de la MCP-2 a través del TGF-β activo unido a la TSP1 y no de la activación del TGF-β latente producido por las células U937.

Usando anticuerpos monoclonales dirigidos contra los dominios N- y C-terminal de la TSP1 deducimos que el dominio N-terminal de la TSP1 puede estar mediando la inhibición de la expresión de las citoquinas IL-6, IL-8, IL-10 y de la quimioquina IP-10 y que el dominio C-terminal de la TSP1 puede estar mediando la inhibición de la expresión de RANTES. Una interpretación alternativa es que el anticuerpo aumenta la estimulación mediada por un dominio diferente de la TSP1 a través del *cross-linking* de la misma. La inhibición en la expresión de la MCP-2 usando un anticuerpo monoclonal dirigido al dominio N-terminal de TSP1 proporciona evidencias de que este dominio está mediando la señalización intracelular que conduce al aumento en la expresión de la MCP-2.

En conclusión, los datos presentados en este estudio demuestran que la estimulación con TSP1 de monocitos humanos diferenciados a macrófagos de tipo M1, aumenta la capacidad de éstos de reconocer y desencadenar la muerte de la célula tumoral *in vitro* a través de la producción de intermediarios reactivos de oxígeno y de la expresión de citoquinas y quimioquinas. *In vivo*, la TSP1 promueve el reclutamiento de macrófagos de tipo M1 al tumor, al tiempo que disminuye el crecimiento tumoral.

Por supuesto, la TSP1 inhibe el crecimiento tumoral a través de su actividad anti-angiogénica, pero los resultados presentados en este trabajo sugieren que la TSP1 puede estar jugando un papel adicional en la inmunidad tumoral al aumentar el reclutamiento y la citotoxicidad de los macrófagos de tipo M1. De este modo, la posibilidad de evadir la vigilancia inmune innata podría justificar el proceso de selección natural que tiende a reducir la expresión de la TSP1 y/o de la TSP2 durante la progresión tumoral (**Modelo II**).

Modelo II. Papel de la Trombospondina-1 en la inmunidad tumoral



B.DISCUSSION

Several studies have suggested that TSP1 plays an important role in the recruitment of monocytes and macrophages to sites of tissue injury or inflammation (1, 2). Here we provide evidence that over-expression of TSP1 in tumors increases macrophage recruitment *in vivo*. Tumor cells produce a range of chemotactic factors for macrophages. MCP-1 stimulates recruitment of macrophages into tumors *in vivo* (3), and decreased expression of this chemokine was reported to limit infiltration of macrophages into an excisional wound in TSP1 null mice (1). However, soluble TSP1 did not increase MCP-1 release from differentiated U937 human monocytic cells.

PAI-1 is also required for cell migration *in vitro* (4), and evidence is emerging for a critical role of PAI-1 in macrophage migration *in vitro* and *in vivo* (5). TSP1 up-regulates PAI-1 in pancreatic cancer cells (6), and PAI-1 expression is up-regulated in TSP1-expressing MDA-MB-435 cells (7). We found that TSP1 acutely increased PAI-1 production by differentiated human and mouse macrophages, and strong TAM expression of PAI-1 was observed in TSP1 over-expressing tumors *in vivo*. This acute change in PAI-1 expression in response to TSP1 is at least partially TGF-β-mediated. Activation of latent TGF-β is mediated by the type 1 repeats of TSP1, but we found no induction of PAI-1 by this domain. Therefore, TSP1 probably induces PAI-1 via bound active TGF-β rather than activation of latent TGF-β produced by U937 cells. Although additional cytokines may be involved, autocrine induction of PAI-1 via tumor-cell produced TSP1 as well as paracrine induction of PAI-1 expression in TAMs could increase macrophage recruitment into the tumor.

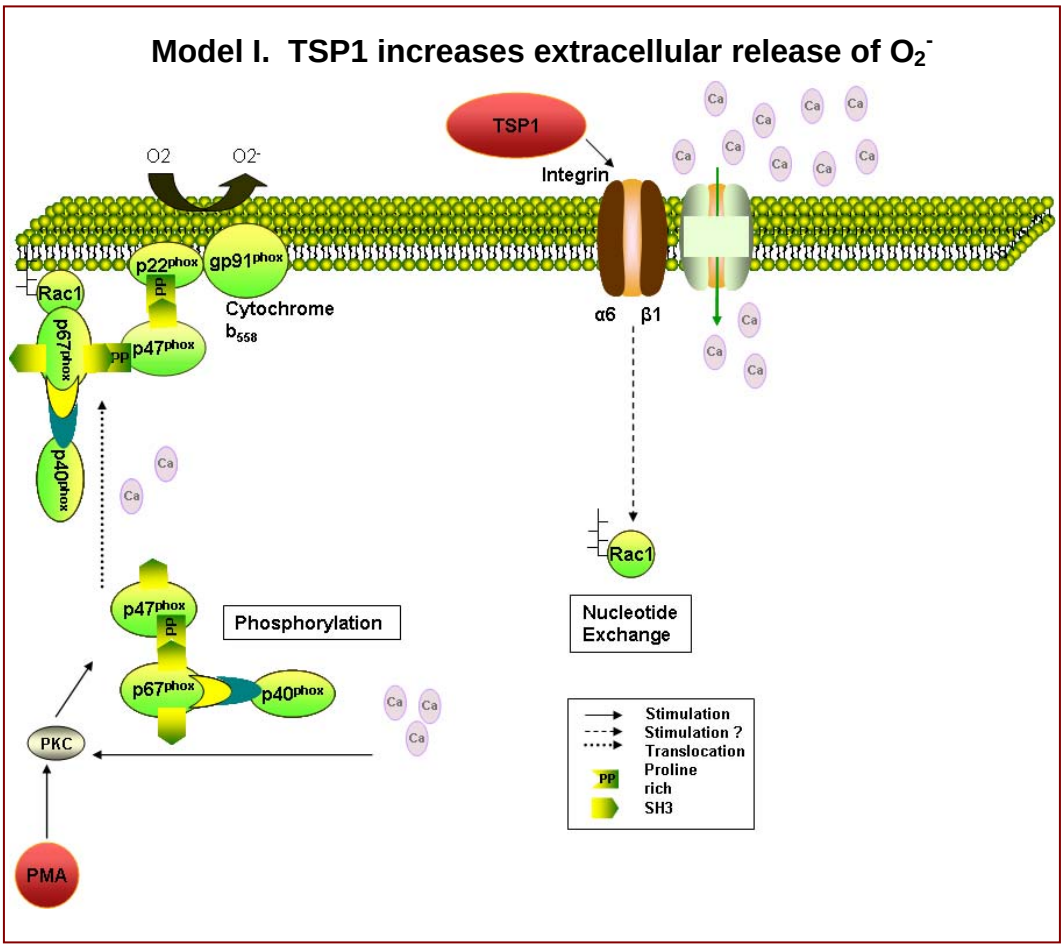
Whether increased macrophage recruitment inhibits or enhances tumor growth depends on their differentiation state. The tumor environment can educate TAMs toward a tumor-promoting phenotype (M2) (8), which may prevent further macrophage migration within the tumor and ensure constant production of growth and angiogenic factors. In addition to contributing to macrophage infiltration by stimulating PAI-1 signaling in TAMs, we found that TSP1 expression in the tumor selectively increases M1 macrophage infiltration. This may provide a selective pressure distinct from its anti-angiogenic activity to account for the frequently observed down-regulation of TSP1 during tumor progression and its ability to inhibit tumor growth when re-expressed.

In general, M1 macrophages are efficient producers of reactive oxygen and nitrogen intermediates that mediate resistance against tumors (9). Here we provide evidence that tumor expression of TSP1 increases M1 polarization of TAMs assessed by iNOS expression.

TSP1 was previously shown to enhance cytokine- and chemoattractant fMLP-induced respiratory burst in human neutrophils (10, 11). We now demonstrate that TSP1 enhances PMA-mediated respiratory burst in U937 cells differentiated along an M1 pathway using IFN-γ. TSP1 stimulates the cytotoxic activity of differentiated human U937 cells and murine ANA-1 cells against several human breast carcinoma and melanoma cell lines. This contrasts with the reported activity of U937 cells to support tumor growth in an M2 manner when co-injected with prostate carcinoma cells (12). We found no effect of TSP1 on M2 differentiation of these cells *in vitro*. Therefore, U937 monocytes have the capacity to differentiate along both pathways, but TSP1 selectively enhances the cytotoxic effector function of M1 macrophages.

The N-terminal domains of TSP1 and TSP2 are sufficient for this priming activity but not for PAI-1 induction. The N-terminal domain of TSP1 mediates interactions with several integrin and non-integrin receptors (13). Differentiated U937 cells express α4β1 (14) and α6β1 integrins, and inhibition of α6β1 using a TSP1 peptide or a function-blocking α6β1 antibody provide evidence that this integrin mediates intracellular signaling pathways leading to increased O_2^- production.

Interactions between integrins and their ligands can trigger a transient elevation in intracellular free Ca^{2+} (15), (16), and Ca^{2+} is a well known intracellular second messenger for signaling the generation of O_2^- in human monocytes (17). We found that addition of TSP1 to differentiated U937 cells caused a significant increase in intracellular free Ca^{2+}, and that chelation of extracellular Ca^{2+} inhibits the stimulatory effect of TSP1 on mobilization of intracellular Ca^{2+} and PMA-mediated O_2^- generation in differentiated U937 cells (**Model I**).



Taken together, these data suggest that a Ca^{2+}-dependent mechanism is involved in TSP1 modulation of the macrophage respiratory burst. However, we can not exclude the possibility that additional receptors recognized by the N-domains of TSP1 might contribute to O_2^- production by macrophages.

It is interesting that the N-terminal region of TSP2 shares this activity to stimulate O_2^- production by macrophages. Loss of TSP2 has also been noted with progression in some cancers, and over-expression of TSP2 limits tumor growth in murine models (18). Our data suggest this may involve both suppression of angiogenesis and enhancement of innate anti-tumor immunity.

Soluble TSP1 at 5 μg/ml was sufficient to increase U937-mediated cytotoxicity in all tumor cell targets tested. TSP1 was also reported to directly induce death of MDA-MB-231 and MCF-7 cells when used at 10 μg/ml for 24 h (19). Our results using a cytotoxicity assay based on LDH release showed minimal cytotoxic activity of soluble TSP1 against MDA-MB-231 cells, even after 72 h of incubation. Our data indicate that TSP1 stimulates macrophage-mediated tumor cell death due to accumulation of ROS. Physiologic doses of O_2^- generated using xanthine/xanthine oxidase were sufficient to inhibit MDA-MB-231 breast carcinoma cell viability.

Classically polarized activated M1 macrophages are not only proficient producers of reactive oxygen and nitrogen intermediates but also efficient producers of inflammatory cytokines (9). TSP1 was previously shown to regulate the release of IL-6 and IL-10 by U937 cells stimulated with PMA and lipopolysaccharide (LPS) (20). We now demonstrate that soluble TSP1 enhances the production of proinflammatory cytokines and chemokines in U937 cells differentiated along an M1 pathway using IFN-γ, and identify NF-κB as a downstream effector.

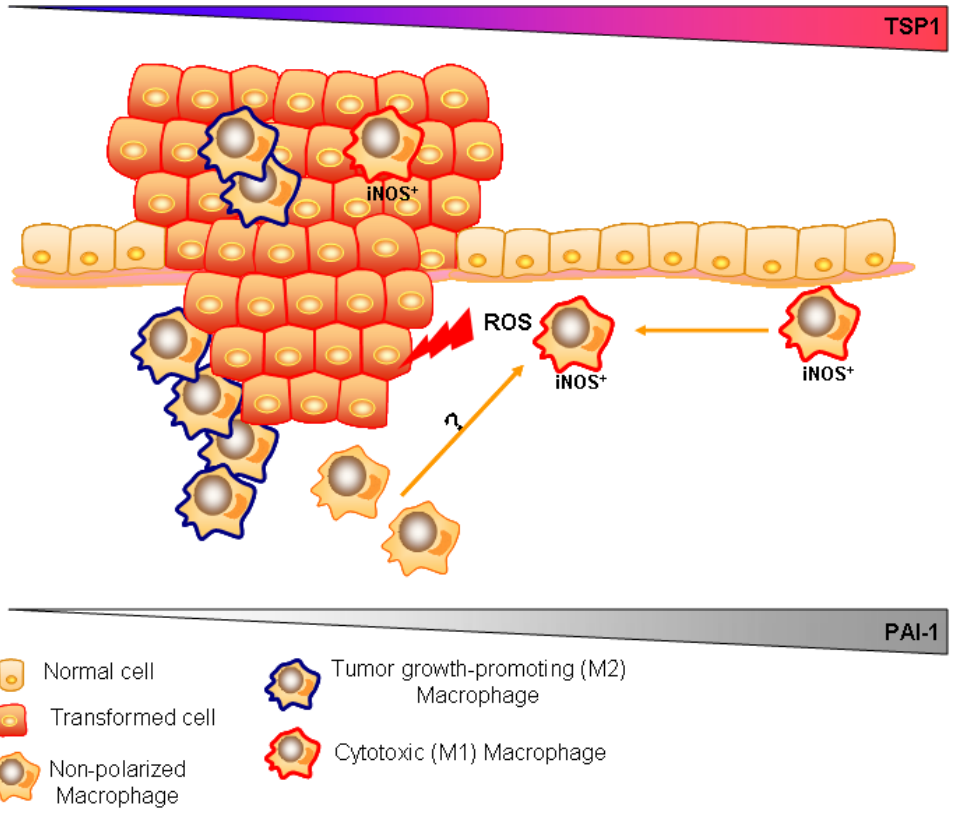
TSP1 stimulated the release of RANTES and IL-8, chemokines involved in mixed leukocyte and neutrophil recruitment, respectively, by IFN-γ-differentiated human U937 cells. Our results using recombinant TGF-β1 and recombinant TSP1 showed negative regulation of these cytokines by bioactive TGF-β1 present in platelet TSP1 (21).

We found that TSP1 acutely increased MCP-2 production by differentiated human macrophages. This acute change in MCP-2 expression in response to TSP1 is at least partially TGF-β-mediated. Activation of latent TGF-β is mediated by the type 1 repeats of TSP1, but we found no induction of MCP-2 by this domain. Therefore, TSP1 probably induces MCP-2 via bound active TGF-β rather than activation of latent TGF-β produced by U937 cells.

Our results using anti-TSP monoclonal antibodies against the N- and C-terminal domains of TSP1 suggest that the N-terminal domain of TSP1 can mediate inhibition of IL-6, IL-8, IL-10, and IP-10 expression, and the C-terminal domain of TSP1 can mediate inhibition of RANTES expression. Alternatively, the antibody may increase a stimulation mediated by a different domain of TSP1 by cross-linking TSP1. Inhibition of MCP-2 expression using an anti-TSP monoclonal antibody against the N-terminal domain of TSP1 provides evidence that this domain mediates intracellular signaling pathways leading to increased MCP-2 expression.

In conclusion, the data presented here demonstrate that stimulation of M1 differentiated human monocytic cells with TSP1 enhances tumor cell killing *in vitro* via production of reactive oxygen intermediates, and cytokine and chemokine expression. *In vivo*, TSP1 promotes M1 macrophage recruitment into tumors while decreasing tumor growth. Clearly, TSP1 can also inhibit tumor growth via its anti-angiogenic activity, but our results suggest that TSP1 plays an additional role in tumor immunity by increasing M1 macrophage recruitment and cytotoxicity. Avoiding this innate immune surveillance could provide a second selective pressure to reduce TSP1 and/or TSP2 expression during tumor progression (**Model II**).

Model II. Role of TSP1 in tumor immunity



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V. CONCLUSIONES

II

A.CONCLUSIONES

1. La sobreexpresión de TSP1 en células de melanoma, aumenta el reclutamiento de macrófagos en tumores xenotrasplantados en ratones atímicos de tipo *nude* y *beige/nude*. *In vitro*, la TSP1 induce significativamente la expresión por parte de monocitos del inhibidor-1 del activador de plasminógeno, lo que sugiere que el reclutamiento de macrófagos en respuesta a la TSP1 está mediado, al menos en parte, por el inhibidor-1 del activador de plasminógeno.

2. Los tumores que sobreexpresan TSP1 presentan un porcentaje más elevado de macrófagos de fenotipo M1 caracterizados por la expresión de la enzima óxido nítrico sintetasa inducible.

3. *In vitro,* la TSP1 aumenta la actividad citotóxica de las células U937 diferenciadas con IFN-γ, induciendo la muerte celular de distintas líneas celulares de cáncer de mama y de melanoma, a través de la producción de especies reactivas de oxígeno.

4. La incubación con TSP1 de monocitos diferenciados y estimulados con PMA, promueve un aumento significativo en la producción de radical superóxido a través de la interacción de su dominio NH2-terminal con el receptor de tipo integrina α6β1. El dominio NH2-terminal de la TSP2 también estimula la producción de radical superóxido en monocitos. La TSP1 requiere la presencia de calcio extracelular para inducir el *respiratory burst* en macrófagos.

5. *In vitro*, la TSP1 induce significativamente la expresión de RANTES, IL-6, IL-8, IL-10, IP-10, I-309 y de la proteína-2 quimiotáctica para monocitos, en células monocíticas U937 diferenciadas a un fenotipo M1. NF-κB es un efector en esta ruta de señalización que conduce a la expresión de citoquinas y quimioquinas en respuesta a TSP1. *In vitro*, la diferenciación y función de los macrófagos de fenotipo M2 no es sensible a TSP1.

La TSP1 juega un papel relevante en la inmunidad anti-tumoral al aumentar el reclutamiento y la activación de los macrófagos de fenotipo M1 asociados al tumor, lo que justifica el proceso de selección natural que favorece la represión en la expresión de TSP1 y TSP2 durante la progresión tumoral.

B.CONCLUSIONS

1. Overexpression of TSP1 in melanoma cells increases macrophage recruitment into xenograft tumors grown in nude or beige/nude mice. *In vitro*, TSP1 acutely induces expression of plasminogen activator inhibitor-1 by monocytic cells, suggesting that TSP1-induced macrophage recruitment is at least partially mediated by plasminogen activator inhibitor-1.

2. The percentage of M1-polarized macrophages expressing inducible nitric oxide synthase is increased in TSP1-expressing tumors.

3. TSP1 stimulates killing of breast carcinoma and melanoma cells by IFN-γ-differentiated U937 cells *in vitro* via release of reactive oxygen species.

4. TSP1 causes a significant increase in PMA-mediated superoxide generation from differentiated monocytes by interaction with α6β1 integrin through its NH2-terminal region. The NH2-terminal domain of TSP2 also stimulates monocyte superoxide production. Extracellular calcium is required for the TSP1-induced macrophage respiratory burst.

5. *In vitro*, TSP1 acutely induces expression of RANTES, IL-6, IL-8, IL-10, IP-10, I-309, and Monocyte Chemoattractant Protein-2 by M1-differentiated U937 monocytic cells. NF-κB is a downstream effector of TSP1-mediated cytokine and chemokine expression. M2 macrophage differentiation or function *in vitro* is not sensitive to TSP1.

TSP1 play an important role in anti-tumor immunity by enhancing recruitment and activation of M1 tumor-associated macrophages, which provides an additional selective pressure for loss of TSP1 and TSP2 expression during tumor progression.

VI. MATERIALS AND METHODS

VI

B.ADDITIONAL MATERIALS AND METHODS

Proteins and peptides. Human TSP1 was purified from the supernatant of thrombin-activated platelets obtained from the NIH Blood Bank (1). Recombinant protein containing the type I repeat domain of TSP1 (3TSR) was prepared as previously described and provided by Dr. Jack Lawler (Harvard University) (2). Recombinant human TSP1 was obtained from EMP Genetech.

Reagents. Monoclonal neutralizing antibody (clone 9016) against human TGFβ1, recombinant human IFN-γ, and recombinant human TGF β1 were from R&D Systems. Anti-TSP mouse monoclonal antibodies Ab-6 (clone A2.5) and Ab-3 (clone C6.7) were from Thermo Scientific. LPS from *Escherichia coli* 0111:B4, PMA and FMLP were obtained from Sigma-Aldrich.

Cell culture and differentiation. The human monocytic line U937 (3) kindly provided by Dr. Mark Raffeld (NCI, NIH, Bethesda, MD) was cultured at 37 ºC, 5% CO_2, in RPMI-1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% endotoxin tested FBS (Biosource). For differentiation with IFN-γ, $2.0x10^5$ U937cells/ml in complete growth medium containing 1mM sodium pyruvate, 0.1mM MEM with non-essential amino acids (Cellgro), and 40-100 U/ml recombinant human IFN-γ were incubated for 3 days at 37ºC.

Real-time quantitative reverse transcription-PCR analysis. Total RNA was extracted from U937 cells using Absolutely RNA Miniprep Kit (Stratagene), according to the manufacturer's instructions. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Nano-Drop Technologies). cDNA was synthesized from total RNA using iScript cDNA Synthesis Kit (BioRad).

Real-time quantitative reverse transcription-PCR analysis was performed on a Bio-Rad iCycler Real Time PCR instrument using the RT^2 Profiler PCR Array for human inflammatory cytokines and receptors (SuperArray Bioscience Corporation). Data were normalized against human β-2-microglobulin (B2M: NM_004048). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Measurement of human cytokines and chemokines. Human cytokines and chemokines levels in differentiated U937 cells supernatants were measured with a Multiplexed ELISA array (Quansys Biosciences). All samples were run in replicate.

Plasmids. PathDetect® cis-Luciferase reporter plasmids containing five copies of the NF-κB binding sites (p NF-κB-luc), and negative control (pCIS-CK-luc), were obtained from Stratagene. *Renilla* Luciferase reporter plasmid (phRL-TK) was obtained from Promega.

Transient Transfection and Luciferase Assay. Differentiated U937 cells were transfected with 1 μg of total DNA (0.8 μg pNF-κB-luc, 0.2 μg phRL-*Renilla*, and 0.8 μg pCIS-CK-luc) in room temperature Cell Line Nucleofector Solution V to a final concentration of $1.0x10^6$ U937cells per 100 μl reaction using Nucleofector (Amaxa biosystems). After transfection, cells were cultured in complete medium for 48 h. Cells were then seeded into 24-well plates in 200 μl of complete medium and stimulated with PMA, LPS, FMLP, and TSP1 for 90 min. Cells were then lysed in passive lysis buffer (Promega), and luciferase activity of protein samples was determined by the Dual-Luciferase® Reporter Assay System (Promega). Luciferase activity was normalized to control vector, phRL-TK.

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